EXTERNAL SCIENTIFIC REPORT

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Review of immune stimulator substances/agents that are susceptible of being used as feed additives: mode of action and identification of end-points for efficacy assessment

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Abstract

Additives may exert a beneficial effect on the immune system and could thus improve the welfare of animals. In most cases, the mode of action of these additives is unknown, which makes assessment of their efficacy difficult.

The project OC/EFSA/FEED/2014/01 aims to identify substances/agents used as feed additives aiming to exert a beneficial effect on the immune system of animals. This project considered 1144 scientific articles, which provided data for around 185 substances/agents with the potential to be used as immune feed additives.

Of the 185 substances/agents detected, 51 were probiotics, 25 were classified as prebiotics, 92 as plant extract, five as animal by-products, and 12 were included as 'other substances'. For each substance / agent, the prevalent mode of action for each animal species, interactions with other dietary compounds, relevant end-points that could demonstrate efficacy, methods for the objective measurement of end-points, risks for the safety of the target animals, consumer, users and the environment, existing legislation in third countries, and the patents published until the time of writing were identified.

Key words: feed additive, gut immunity, probiotic, prebiotic, immunostimulation, immunomodulation

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Summary

The project OC/EFSA/FEED/2014/01 seeks to gather information on the potential for substances and agents which, when used in animal feed, could improve immune status or play a role in immunity stimulation as indicated in the tender.

In order to accomplish these objectives, a systematic review was carried out. Fifteen chain searches were run in the three different databases (WoS, Medline, and SciELo) encompassing dates from 1st January 1990 until 12 November 2014. Search chains with specific key words were formulated in order to include as many studies as possible and to avoid the exclusion of any agent/substance with immunomodulatory effects. The number of outputs obtained through the searches was 26301. All duplicate studies and references without full text were excluded from the project due the impossibility of analysing and characterising them. The number of references found with full text attached was 12723.

In order to efficiently manage all articles with full text, the tool Distiller SR© systematic review software was used. Results were imported to Distiller through the EndNote programme. This software allows reference information to be shared by entire review teams. Data fields within the reference records were used to retain decisions (e.g. about study inclusion or exclusion) or queries about the publication. After the inclusion / exclusion process, 1144 papers were considered valid for inclusion in the review.

First, the papers were sorted by target species and type of substances (probiotic, prebiotic, plant extract, animal by-product, and other substances / agents). For each substance / agent, the prevalent mode of action for each animal species, interactions with other dietary compounds, relevant end-points that could demonstrate the efficacy, methods for the objective measurement of the end-points, risks for the safety for the target animals, consumer, users, and the environment, existing legislation in third countries, and patents published until the time of writing were identified.

Due to the emergence of microbes which are resistant to antibiotics used to treat human and animal infections, the European Commission (EC) decided to ban the use of antibiotics as growth promoters (AGP) in animal feed. Alternatives to growth promoters should have the

same beneficial effects as AGPs but precisely how AGPs exert beneficial action is currently unclear. The most well-known mechanism to be proposed is that AGPs have an antibacterial action that improves performance in different ways: (1) by reducing the incidence and severity of subclinical infections; (2) by reducing the microbial use of nutrients; (3) by improving absorption of nutrients due to thinning of the intestinal wall, (4) by reducing the amount of growth-depressing metabolites produced by Gram-positive bacteria and (5) by inhibiting inflammatory response.

The focus of alternative strategies (i.e. the addition of immunomodulatory substances/agents in animal diets) has been thought to prevent the proliferation of pathogenic bacteria; it is also thought to affect the modulation of commensal bacteria, modulation of the local and systemic immune response, and to improve animal welfare and the health status of animals.

Probiotics have a wide variety of effects on the immune system. These effects depend on probiotic strain, animal species, dose, time, and mode of administration. Generally, the use of probiotics in different species focuses on avoiding pathogen infection/proliferation and on improvement of animal welfare. Probiotic compete with pathogenic bacteria in adherence to the mucus layer covering the intestinal epithelium, interfering with the pathogen colonisation in the gut and, in most cases, modulating the host immune response. Intestinal morphology can be affected by dietary supplementation of different immunomodulatory substances / agents. The cellular components of innate immune system (macrophages and heterophils) protect the host from enteric infection. When intestinal microorganisms breach the intestinal epithelial barrier, these immune cells are recruited to the site of infection, where they kill the invaders using a variety of strategies, such as phagocytosis and oxidative burst. After toll-like receptor (TLR) activation, one possible outcome is the synthesis and release of pro-inflammatory cytokines. The presence of these cytokines modulates adaptive immunity. The manipulation of gut microbiome through administration of immunomodulatory substances / agents can influence cell- and antibody-mediated immune response. The main target for immunomodulatory feed additives is the reduction of local inflammation and limitation of further impairment of immune function.

Due to their different origins, characteristics, and properties, prebiotics exert different effects on the organism and, unlike with probiotics, generalising their mode of action is not simple. Some prebiotics modulate intestinal microbial communities (increasing total aerobes bacteria and decreasing enterococci), which subsequently improved gut morphology and the epithelial brush border. Generally, the administration of prebiotics in feed directly affected the gross morphology of the intestine by promoting its development and increasing the intestinal barrier. Another mode of action detected using prebiotics is the enhancement of innate immune factors by up-regulating expression of complement factors and acute phase proteins. The modulation of pro- and anti-inflammatory cytokines is involved in the induction of cellular and humoral immune system.

Plant extracts or phytogenic feed additives comprise a large number of compounds and a variety of herbs, spices, and products derived thereof, particularly essentials oils. A substantial number of studies concern plant extracts. The mode of action of these plant extract is poorly investigated either because these were studied primarily with regard to mixtures or because only a few studies are available. Plant extracts can promote the growth of bacterial species that are potentially beneficial to the host and promote an increased concentration of SCFA in the ileum and colon while reducing potentially harmful bacteria and the production of protein-derivative catabolites. Plant extracts have different compounds, including molecules with immunostimulant properties, and are able to modulate cellular and humoral immunity. However, the effect on the immune system is specific to each compound, and it is necessary to assess the mode of action individually.

Animal by-products refer to entire bodies or parts of animals, products of animal origin, or other products obtained from animals. The effect of these products on performance is not clear, but immune modulation has been described. It seems that the use of these products can result in the enhancement of the humoral immune response, which translates into increased protection from pathogens.

All feed additives not able to be classified into the previous groups were included in a wide group called 'other'. This classification includes vitamins, amino acids, organic acids, minerals, enzymes, algae, etc. The modes of action of these substances are varied, and it is

necessary to assess their mechanisms on an individual basis. Generally, these products have beneficial effects on performance, gut structure, and the modulation of immune system.

In order to describe the benefits of the application of substances or agents on the immune system, the end-points assessed in the scientific articles were classified intothree main groups for the majority of animal species: local immune response, systemic immune response, and health status; with the exception of fish,the end-points were classified as measured immunological parameters and health status. The most evaluated parameters were intestinal microbiota and gut structure (local immune response), immunoglobulins and cytokines (systemic immune response), and performance (health status). In the case of fish, the parameters that were evaluated the most were lysozyme activity, leucocyte count, complement activity, immunoglobulin quantification, respiratory burst and phagocytic activity (immunological parameters studied), and performance (health status).

Another parameter considered was the interaction between the presence of additive and other dietary components. In this revision, the main interactions studied were the ones between two or more immunomodulatory substances / agents added to the diet; in fact, few interactions between dietary composition and substances/agents were identified.

This systematic search also includes a specific section in which safety for the target animals, consumers, users, and environment was assessed. A small number of studies evaluated the safety of the additives; this is likely because the main purpose of this systematic review was to identify substances /agents with immunomodulatory effects that might make it possible to use themin feed additives. The evaluation of the safety of these substances was not the primary goal of the research.

Finally, a worldwide patent search was conducted for each substance/agent included in this study; this is thought to compile all the protected knowledge published until the point of writing.

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1. Introduction

1.1. Background and terms of reference as provided by the requestor

The scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) assesses the safety and efficacy of feed additives.

Regulation (EC) No 1831/2003(Regulation, 2003) establishes the rules for the authorisation of feed additives in the European Union (EU). Applicants wishing to place a feed additive in the EU market shall send an application to the European Commission (EC) and a technical dossier to EFSA. It is the task of EFSA to provide the ECwith a scientific opinion in which the assessment of the safety and the efficacy of the feed additive is reported. This task is entrusted to the FEEDAP Panel. The FEED Unit provides scientific and technical / administrative support to the Panel.

Article 5(3) of the aforementioned Regulation provides the definition of efficacy of feed additives. In particular, it lays down that no feed additive shall be authorised unless it has been demonstrated that it satisfies one or more of the following conditions.

- Favourably affect the characteristics of feed, animal products, and/or the colour of ornamental fish and birds;
- Satisfy the nutritional needs of animals;
- Favourably affects the environmental consequences of animal production;
- Favourably affects animal production, performance or welfare, particularly by affecting the gastrointestinal flora or digestibility of feeding-stuffs;
- Have a coccidiostatic or histomonostatic effect.

Additives that may exert a beneficial effect on the immune system of animals would, in principle, fulfil the above mentioned criteria by improving the welfare of animals. In recent years, the number of applications for feed additives aiming to exert a beneficial effect on the immune system of animals, especially companion animals, has increased considerably. However, in most of the cases the mode of action of these additives is unknownwhich makes assessment of their efficacy difficult.

Regulation (EC) No 429/2008 (Regulation, 2008a) includes general provisions for proving the efficacy of feed additives. Efficacy studies should be designed to demonstrate the effect(s) of the additive by targeting sensitive parameters in comparison to a negative and,optionally, a positive control group. The FEEDAP Panel has issued a series of guidance documents to help the applicants in the preparation of dossiers. In these guidance documents, the provisions for demonstration of efficacy required by Regulation (EC) No 429/2008 are described in more detail. No specific guidance from the FEEDAP Panel exists for the assessment of this type of feed additives.

This contract/grant was awarded by EFSA to:
Contractor: **Institut de Recerca i Tecnologia Agroalimentàries (IRTA)**
Contract: **Review of immune stimulator substances/agents that are susceptible of being used as feed additives: mode of action and identification of end-points for efficacy assessment**
Contract/Grant number: **OC/EFSA/FEED/2014/01**

2. Data and Methodologies

2.1. Searches

In order to identify all substances/agents with the potential to be used as feed additives with a direct favourable effect on the immune system of animals, an extensive and systematic examination of publications and relevant patents has been undertaken.

To retrieve optimal results, the literature search was conducted using four different databases (three bibliographies and one patent database):

- **Web of Science Core Collection (WoS)** contains information gathered from thousands of scholarly journals in all disciplines and more than 250 fields of study, including agriculture, animal, biological, environmental and veterinary sciences.
- **MEDLINE** provides authoritative medical information on medicine, veterinary medicine, the health care system, pre-clinical sciences, etc.
- **SciELO Citation Index** has nearly 650 journals and over 4 million cited references centred in Latin America, Spain, Portugal, the Caribbean and South Africa.
- **Questel-Orbit**© is the world's most comprehensive patent database.

2.1.1. Search chains (databases)

In order to conduct the searches in the three databases mentioned above (WoS, Medline, and SciELO), the website belonging to Web of Science was used (www.webofknowledge.com). This allowed all searches to be saved, with outputs obtained and the date to guaranteeing traceability. The range of dates searched included 1st January 1990 until 12th November 2014 (with the exception of SciELO, for which the database has only been available since 1997).

Search chains with specific key words were formulated in order to include as many studies as possible and avoid the exclusion of any agent/substance with immunomodulatory effects.

Fifteen search chains were run in the three databases in order to maximize output. The search chains used are shown in Table 1.

Table 1: Search chains

	Search chains
1	immune feed additive
2	oral immunomodulation gut animal
3	food immune system intestinal animal
4	oral immune system intestinal animal
5	immunomodulation feed
6	immunostimulation feed
7	food immune gut animal
8	oral immune gut animal
9	immune gut feed
10	immune intestinal feed
11	immunity gut feed
12	immune intestinal food animal
13	immune additive animal
14	Prebiotic
15	probiotic* *OR* prebiotic* *AND* action *AND* immun* *AND* animal *AND* intestin* *AND* YEAR PUBLISHED 1990-2014 *AND* feed* NOT allerg* *NOT* human)) *NOT* cancer*

2.2. Selection

The selection process is divided into the following steps (see Figure 1):

STEP 1: Results obtained with all searches were imported to an Endnote file (EndNote is a bibliographic software program for managing references). They were then organised by database and search chain. Duplicates were removed duringthis step.

STEP 2: All full texts available to the institution were downloaded, and references without full text were removed from the project due the impossibility of analysing and characterising them.

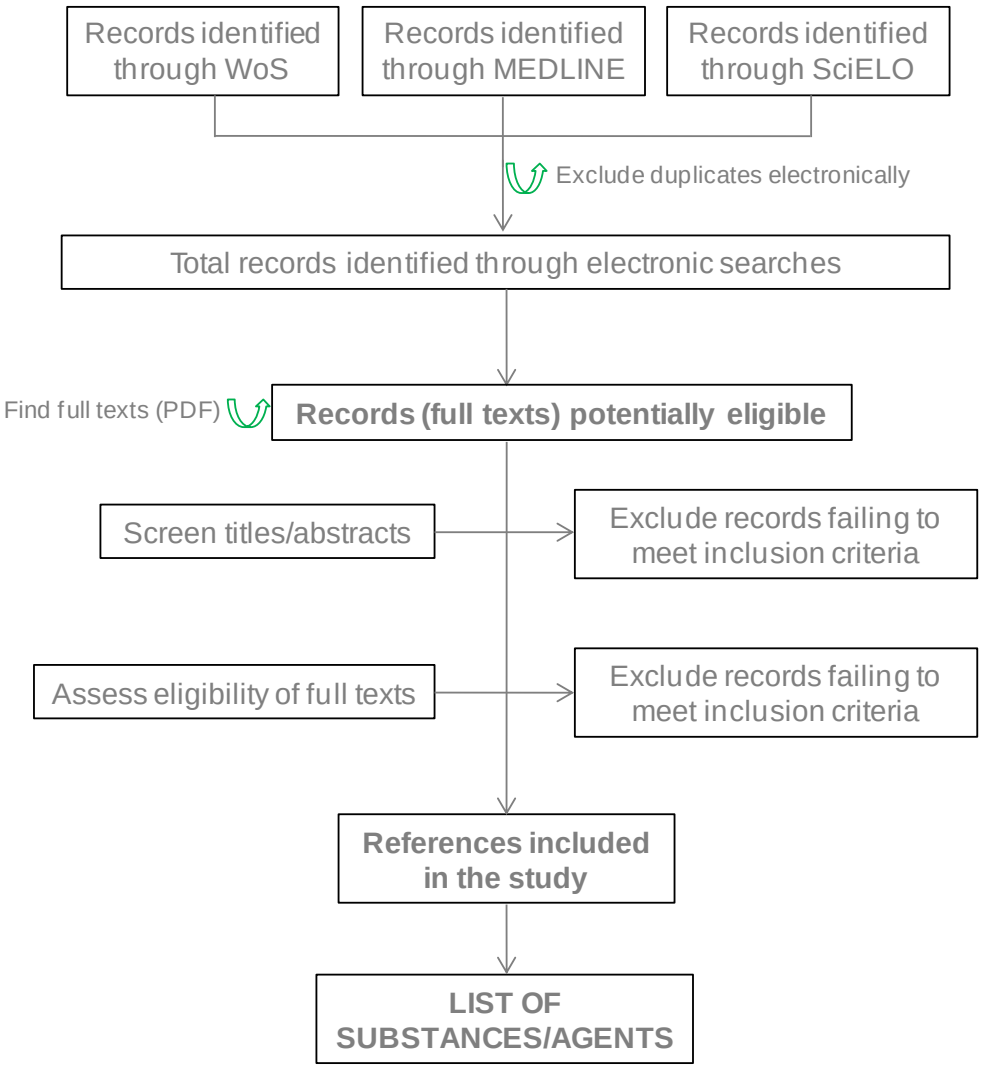
STEP 3: In order to manage all articles with full text, the tool Distiller SR© systematic review software was used. Results were imported to Distiller through the EndNote program. This software allows reference information to be shared by the whole review teams. Data fields within the reference records were used to retain decisions (e.g. about study inclusion or exclusion) or queries about the publication.

STEP 4: Selection based on **title and abstract**. The first step, using software Distiller, was to include or exclude all references analysing title and abstract **(level 1 and 2)**. Two members of the team independently checked the set of articles, and decided whether the initially selected articles should be included or excluded, by applying the inclusion/exclusion criterion of key elements on titles and abstracts. In this step, the inclusion criterion was that the study was related to the tender (feed additive with direct effect on immune system). Included articles, excluded articles, and articles classified as doubtful recorded in the data collection form. Included articles were incorporated directly into Level 3 of Distiller for their review. If a team member was not sure whether to include or exclude an article, Level 1 allowed classifying this work as a doubtful. The doubtful articles were included into level 2 of Distiller. These works were discussed in a meeting with the other team member, and a decision was taken. Level 2 had the same inclusion/exclusion criteria than Level 1 but only allowed to include or exclude the article of the review process. If screening through title and abstract was unclear, the full text was retrieved.

STEP 5: Selection based on **full text (level 3)**. The relevant or suspicious articles previously selected on the basis of title and abstract screening (level 1 and 2) , were screened to be included or excluded based on the full text, attending the inclusion/exclusion criteria set up in the protocol. Inclusion criteria guarantee the quality of each study.

Included and excluded articles were recorded in the data collection form. The studies excluded in this step were reported, explaining the reasons in detail. If required (for instance, disagreements between reviewers occur), independent reviewers helped to resolve differences in opinion.

Figure 1: Scheme of search and selection process.



2.3. Inclusion / exclusion criteria

The criteria used to include or exclude the articles in level 1 and 2 (Distiller SR) are described below (Table 2):

LEVEL 1 and 2 (Distiller SR)

Table 2: Inclusion and exclusion criteria level 1 and 2 (Distiller SR)

INCLUSION CRITERIA	EXCLUSION CRITERIA
Reference with full text (PDF)	Reference without full text (PDF)
Research article	Non-research article
Target species described in the tender specifications	Target species not described in the tender specifications
Feed additive with direct effect on immune system	Feed additive without direct effect on immune system or not a feed additive

LEVEL 3 (Distiller SR)

The inclusion criteria established in the level 2 of Distiller were developed to guarantee the methodological **quality** of each article. The quality assessment checklist analyses aspects of the design, execution, analysis, and reporting of a study. These criteria established the standards and conditions that each study should meet in accordance with the aims and objectives of the project.

The inclusion criteria depend of the type of experiments (*in vivo, in vitro,* or *ex vivo*) and are divided into three groups:

i) **Essential conditions:** These must be met in full for each study. Failure to meet the criteria meant that the study was not included in the review.

ii) **Highly Desirable conditions:** If two of more of these conditions were not met, the study was rejected.

iii) **Desirable:** If three of more of these conditions were not met, the study was rejected.

Table 3 illustrates thequality assessment checklist used in this project.

Table 3: Quality assessment checklist used in level 3 (Distiller SR), according to type of study

ESSENTIAL CONDITION	*IN VIVO*	*IN VITRO*	*EX VIVO*
Is the study clearly written?	✓	✓	✓
Has a negative control been used?	✓	✓	✓
Has appropriate statistical analysis been undertaken?	✓	✓	✓
Have the diets and supplementation been clearly described?	✓	✓	✓
Do the conclusions fully support data?	✓	✓	✓
Do the end-points answer the both specific aims and objectives?	✓	✓	✓
Livestock are described by species AND numbers?	✓	×	×
A clear description of methods used to collect samples and analyse the data has been given.	✓	×	×
Are the cell type used well characterized?	×	✓	×
Has the incubation period been reported?	×	✓	×
Has the incubation temperature been recorded within the report?	×	✓	×
Has culture composition and preparation been adequately described within the report?	×	✓	×
Have a minimum of two replicates been analysed for each in vitro sample?	×	✓	×

HIGHLY DESIRABLE CONDITION	*IN VIVO*	*IN VITRO*	*EX VIVO*
Are the results presented in such a way that interpretation is simple and conclusions can be verified?	✓	✓	✓
Are the aims, objectives and context of the study clearly stated an appropriated?	✓	✓	✓
Livestock are described by weight AND/OR age.	✓	×	×
Are treatment groups processed identically through the entire experiment?	✓	×	×
Is the animal sample size adequate for the study?	✓	×	×
Has the number of donors been described?	×	✓	×

DESIRABLE CONDITION	*IN VIVO*	*IN VITRO*	*EX VIVO*
Does the study include an adequate literature review and is it adequately referenced through?	✓	✓	✓
Are the experimental groups located to avoid unnecessary variations?	×	✓	×
Is the immune stimulator dose commensurate with a normal expected dose?	✓	✓	✓
Does the study state specifically that best practice has been used including full compliance with national/local regulations?	✓	×	×

Documents that satisfied the quality assessment were then subject to the review process.

2.4. Review process

The international expert advisory panel and all groups involved explorated how scientific analysis should be conducted.

The decisions taken are indicated in Figure 2, which illustrates all the information in order to collate each research article selected in Level 1 of Distiller SR and comply with the appropriate quality assessment in Level 2 of Distiller.

Figure 2: Scheme of data analysis

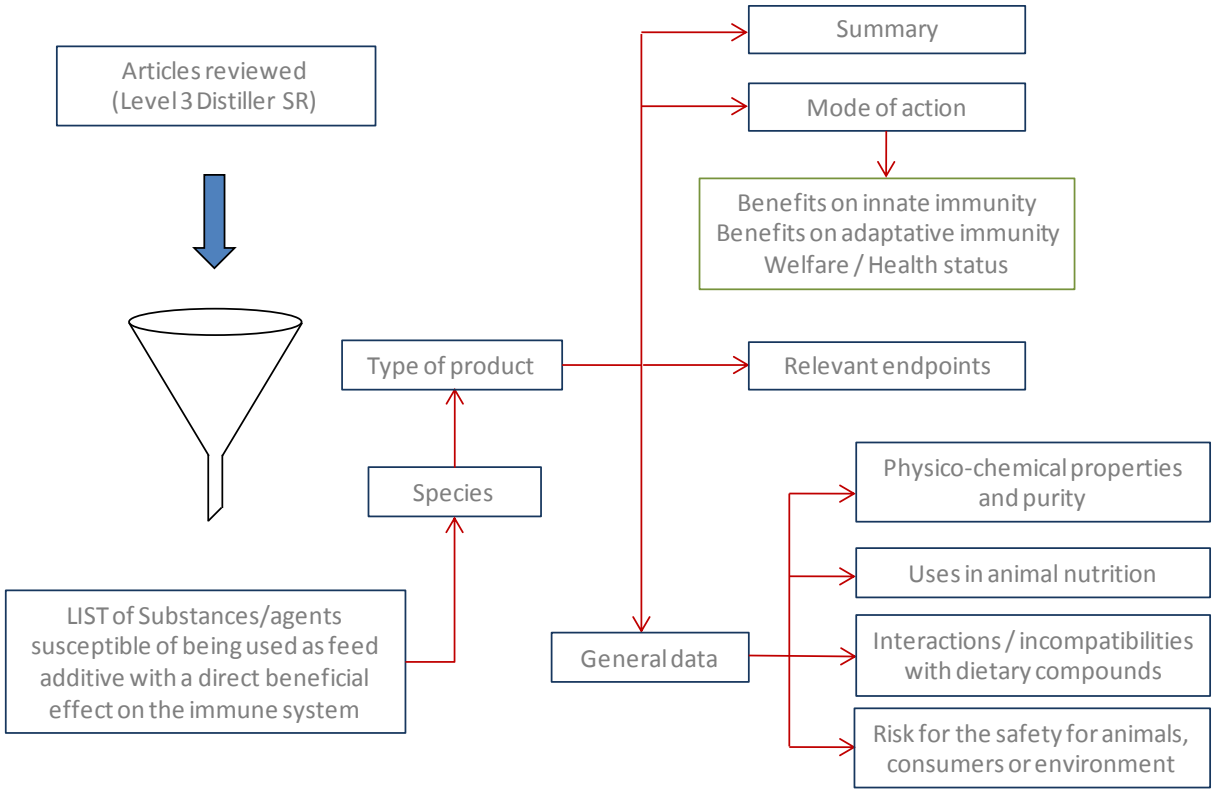


Table 4 describes the entire questionnaire relevant to the selected articles (Level 3 of Distiller SR). This questionnaire allows readers to obtain answers for all parameters described in Figure 2. The Distiller SR software allows conduction of this survey and is able to store all relevant information. Using this programme, data is easily manipulated, allowing for the creation of a specific table of a selected parameter.

Table 4: Complete questionnaire of Level 3 of Distiller SR (after inclusion criteria).

Question Text	Answer Text
Study type	In vitro
	In vivo
	Ex vivo

Question Text	Answer Text
Substance/agent	Probiotic
	Prebiotic
	Plant extract
	Animal by-product
	Other
This form is legislated by EC?	Yes
	No
	Other legislations
	Information not provided
Animal category	1.1. Pigs. Piglets (suckling)
	1.2. Pigs. Piglets (weaned)
	1.3. Pigs. Pigs for fattening
	1.4. Pigs. Sows for reproduction
	1.5. Pigs. Sows, in order to benefit piglets
	2.1. Poultry. Chickens for fattening
	2.2. Poultry. Chickens reared for laying
	2.3. Poultry. Laying hens
	2.4. Poultry. Turkeys for fattening
	2.5. Poultry. Turkeys reared for breeding
	2.6. Other Poultry (e.g. ducks, goose, pheasants, quails...)
	3.1. Bovines. Calves for rearing
	3.2. Bovines. Calves for fattening
	3.3. Bovines. Cattle for fattening
	3.4. Bovines. Dairy cows for milk production
	3.5. Bovines. Dairy cows for reproduction
	4.1. Sheep. Lambs for rearing
	4.2. Sheep. Lambs for fattening
	4.3. Sheep. Dairy sheep
	4.4. Sheep. Ewes for reproduction
	5.1. Goat. Kids for rearing
	5.2 Goat. Kids for fattening
	5.3. Goat. Dairy goats
	5.4. Goat. Goats for reproduction
	6. Other Ruminant (i.e. buffalo)
	7.1. Fish. Salmonids (i.e. salmon, trout)
	7.2. Fish. Other freshwater fish (i.e. carp, tilapia, sturgeon)
	7.3. Fish. Other marine fish
	8.1. Rabbits: Rabbits suckling and weaned
	8.2. Rabbits: Rabbits for fattening
	8.3. Rabbits: Rabbits for reproduction

Question Text	Answer Text
	8.4. Rabbits: Rabbits in order to benefit young rabbits
	9. Horses
	10.1. Pets and other non food-producing animals. Dogs
	10.2. Pets and other non-food producing animals. Cats
	11. Fur animals
	12. Other animal categories
Total number of animals and number of animals per treatment group	
Number of replicates per treatment	
Description of treatments, including control (enter dose and concentration units)	
Concentrations are expressed in...	Diet - DM
	Diet - FM
	Diet - CFUs
	Diet - Percentage
	Diet - Other
The product specification was....	Analysed
	Estimated
	Not specified
Duration of the treatment	
Immunological parameters studied (fish)	Phagocytic activity
	Complement activity
	Leucocyte count
	Immunoglobulin quantification
	Haematocrit
	Respiratory (oxidative) burst
	Phosphatase activity
	Protease activity
	Phenoloxidase activity
	Lysozyme activity
	Peroxidase activity
	Bacterial activity
	Intestine morphology
	Other
• Local immune responses (intestine):	Microvilli structure: length of microvilli and crypts
	Oxidative stress
	Cytoquine responses
	Immunoglobulins
	Phenotypic changes in lymphoid cells
	Intestinal microbiota
	Metabolomic studies

Question Text	Answer Text
	Others
• Systemic immune responses (blood/serum):	Acute phase proteins
	Immunoglobulins
	Cytokines
	Phagocytic activity of lymphoid cells
	Cytolytic activity in lymphoid cells
	Lymphocyte proliferation activity
	Phenotypic changes in lymphoid cells
	Weight lymphoid organs
	Other
Effects on immune system	
Mode of action	
• Health status	Diarrhoea
	Mortality
	Morbility
	Performance
	Carcass traits
	Skeleton
	Other
	Not defined
In the study are assessed...	Interactions
	Incompatibilities
	Other
Interaction outcome (enter the parameter by means of which the interactions and/or incompatibilities are assessed)	
The method to assess the interaction/incompatibility is...	Qualitative
	Quantitative
	Other
Enter the name of the combinations assessed	
The effect of the interaction/incompatibility is...	Synergistic
	Additive
	Less than additive
	Antagonistic
	Other
Safe for consumers	Yes
	No
	Not specified
Safe for target animals	Yes
	No

Question Text	Answer Text
	Not specified
Safe for the environment	Yes
	No
	Not specified
Information on...	Potential contaminants/impurities
	Virulence determinants for microorganisms
	Possible metabolites of toxicological relevance
	Irritation/sensitisation potential
	Other information
Enter comments / conclusions	
After reading the full-article, this document is....	INCLUDED
	EXCLUDED
	DOUBTFUL
Exclusion reason	Not about immune modulator substances/agents
	Other reason

2.5. Patents

When the final list of products was obtained, a specific patent search for each product was conducted. In order to carry out this search, Questel-Orbit© software was used. This software allows a worldwide search of patents and classifies each patent by country.

Questel-Orbit is the world's most comprehensive patent database, gathering more than 100 databases for specialists in patents and designs. FamPat, the main patent database, covers more than 95 offices, including China, India, and Japan; these are grouped by invention-based families and enriched with full-text databases.

The searches were conducted into the "cooperative patent classification" (CPC) A23K(Fodder / Animal feeding-stuffs). The following chain search was used: (+IMMUN+)/TI/AB/IW/CLMS AND (**NAME OF THE PRODUCT**)/TI/AB/IW/CLMS) AND (A23K+)/IPC AND PD >= 1995-09-01. This chain of searches allows the identificationofall patents forthe given substance / agent, including the word "immune" (and all derivatives of this word) into the category A23K, from 1st of September of 1995 until the time of writing.

2.6. Legislation in third countries

The EFSA provided the researchers with a list of contacts of international agencies and authorities (e.g. Food Standards Australia New Zealand (FSANZ), New Zealand Food Safety Authority (NZFSA), Canadian Food Inspection Agency (CFIA), United States Food and Drug Administration (US FDA), United States Environmental Protection Agency (US EPA), Food Safety Commission Japan (FSCJ), and others) so that we could request information about product legislation and registration in countries outside of the European Union (EU).

3. Results

3.1. Searches

The number of outputs obtained by the searches is summarised in Table 5.

Table 5: Output obtained after runningthe chain searches in three different databases

	Search chains	Database	Nº results	Duplicates	Total	With full text
1	immune feed additive	Medline	168	17	151	130
2	oral immunomodulation gut animal	Medline	122	2	120	103
3	food immune system intestinal animal	Medline	414	46	368	305
4	oral immune system intestinal animal	Medline	320	67	253	194
5	immunomodulation feed	Medline	579	41	538	383
6	immunostimulation feed	Medline	371	356	15	15
7	food immune gut animal	Medline	414	229	185	158
8	oral immune gut animal	Medline	306	149	157	133
9	immune gut feed	Medline	467	332	135	95
10	immune intestinal feed	Medline	440	362	78	65
11	immunity gut feed	Medline	261	187	74	66
12	immune intestinal food animal	Medline	281	266	15	13
13	immune additive animal	Medline	187	88	99	77
14	Prebiotic	Medline	1200	468	732	508
15	probiotic* *OR* prebiotic* *AND* action *AND* immun* *AND* animal *AND* intestin* *AND* **YEAR PUBLISHED** 1990-2014 *AND* feed* NOT allerg* *NOT* human)) *NOT* cancer*	Medline	9778	2310	7468	5157
			15308		10388	7402

	Search chains	Database	Nº results	Duplicates	Total	With full text
1	immune feed additive	WoS	360	145	215	136
2	oral immunomodulation gut animal	WoS	6	0	6	3
3	food immune system intestinal animal	WoS	128	113	15	13
4	oral immune system intestinal animal	WoS	174	23	151	120
5	immunomodulation feed	WoS	365	66	299	208
6	immunostimulation feed	WoS	105	31	74	56

7	food immune gut animal	WoS	179	52	127	84
8	oral immune gut animal	WoS	272	132	140	116
9	immune gut feed	WoS	1229	408	821	618
10	immune intestinal feed	WoS	1750	978	772	589
11	immunity gut feed	WoS	574	381	193	156
12	immune intestinal food animal	WoS	288	189	99	65
13	immune additive animal	WoS	287	150	137	97
14	Prebiotic	WoS	1625	240	1385	989
15	probiotic* *OR* prebiotic* *AND* action *AND* immun* *AND* animal *AND* intestin* *AND* **YEAR PUBLISHED** 1990-2014 *AND* feed* NOT allerg* *NOT* human)) *NOT* cancer*	WoS	3401	399	3002	2001
			10743		7436	5251

	Search chains	Database	Nº results	Duplicates	Total	With full text
1	immune feed additive	SciELO	6	5	1	1
2	oral immunomodulation gut animal	SciELO	0	0	0	0
3	food immune system intestinal animal	SciELO	0	0	0	0
4	oral immune system intestinal animal	SciELO	0	0	0	0
5	immunomodulation feed	SciELO	1	1	0	0
6	immunostimulation feed	SciELO	3	2	1	1
7	food immune gut animal	SciELO	1	1	0	0
8	oral immune gut animal	SciELO	0	0	0	0
9	immune gut feed	SciELO	6	5	1	1
10	immune intestinal feed	SciELO	15	11	4	3
11	immunity gut feed	SciELO	1	1	0	0
12	immune intestinal food animal	SciELO	1	1	0	0
13	immune additive animal	SciELO	3	2	1	1
14	Prebiotic	SciELO	106	27	79	62
15	probiotic* *OR* prebiotic* *AND* action *AND* immun* *AND* animal *AND* intestin* *AND* **YEAR PUBLISHED** 1990-2014 *AND* feed* NOT allerg* *NOT* human)) *NOT* cancer*	SciELO	107	106	1	1
			250		88	70
		TOTAL	**26301**		**17912**	12723
						With full text

STEP 1: The 26301 references obtained through all searches were imported to the Endnote file. They were organised by database and search chain. In this file, the duplicates were removed both automatically and manually. The total number of references used was 17912.

STEP 2: All full texts available for the institution were downloaded. The number of references with full text found was 12723. All the references without full text were removed from the project due the impossibility of analysing and characterising them. To ensure traceability, each reference includes an annotation with its name and the date on which it was obtained.

STEP 3: In order to manage all articles with full text, the tool Distiller SR© systematic review software was used. The 12723 references with PDF were imported to Distiller through the EndNote programme.

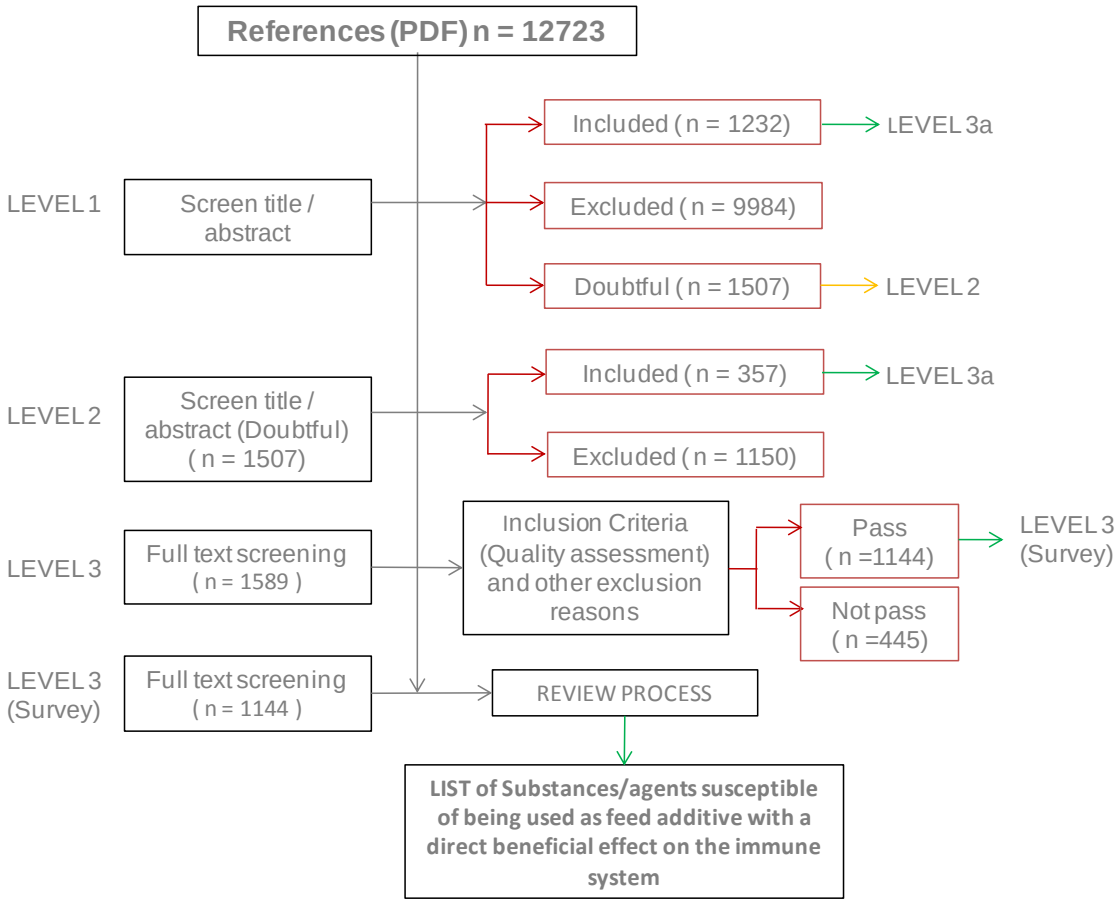
3.2. Selection

STEP 1: Selection was based on title and abstract. The first step involved in using software Distiller was to include or exclude all references and analysing the title and abstract **(levels 1 and 2)**. Two members of the team independently checked the set of articles in order to decide whether or not those initially selected should be included; they did so by applying the inclusion/exclusion criterion pertaining to the key elements of the titles and abstracts. At this stage, the inclusion criterion was whether or not the study was related to the tender (feed additive with a direct effect on the immune system).

STEP 2: Selection based on **full text (level 3)**. The relevant articles previously selected on the basis of their titles and abstracts (level 1 and 2) were screened in order to be included or excluded based on the full text, taking into account the inclusion/exclusion criteria agreed in the protocol. Inclusion criteria guaranteed the quality of each study, and the articles included after quality assessment were analysed in order to fill in the questionnaire described in the Table 3.

Figure **3** describes the entire selection process as well as the number of articles included/excluded at each step.

Figure 3: Scheme of selection



3.3. Substances / agents identified

3.3.1. Probiotics

3.3.1.1 Definition of "Probiotic"

General definition: Probiotics have been defined as mono- or mixed cultures of living microorganisms that beneficially affect the host by improving the properties of the indigenous microbiota' (Fuller, 1992). The available probiotics can be classified into (1) 'colonising' species, such as Lactobacillus and *Enterococcus spp.*, and (2) free flowing 'non-colonising' species, such as *Bacillus spp.* (spores) and *Saccharomyces cerevisiae* (Huyghebaert, Ducatelle, & Van Immerseel, 2011).

Definition as pertaining to feed regulations: Probiotic is also defined as Direct Feed Microbial (DFMs). DFM is a microbial included in the group of feed additive for stabilising the microbial communities of the digestive tract in monogastrics and ruminants.

Probiotics are generally considered to enhance the intestinal microbial balance. However, today there is another important benefit for the host,as there isgrowing evidence that probiotics can modulate the immune system.

3.3.1.2 List of substances / agents (Probiotics)

Table 6: Type of probiotics studied across different animal species

Substance / Agent	Number of articles	Porcine	Poultry	Bovine	Caprine & Ovine	Fish	Rabbits	Equine	Pets
Lactobacillus spp.	274	69	113	14	6	54	2	3	13
Bacillus spp.	159	31	59	6	1	58	2	0	2
Enterococcus spp.	114	29	53	10	2	9	1	2	9
Saccharomyces spp.	87	30	29	4	3	16	3	2	0
Bifidobacterium spp.	65	7	47	2	1	0	1	1	6
Pediococcus spp.	35	11	10	3	0	10	1	0	0
Streptococcus spp.	32	1	26	1	1	1	0	0	2
Aspergillus spp.	26	5	16	1	1	2	1	0	0
Clostridium spp.	14	0	9	3	0	2	0	0	0
Candida spp.	15	0	9	2	0	2	1	0	1
Lactococcus spp.	14	0	1	0	0	12	1	0	0
Aeromonas spp.	7	0	0	0	0	7	0	0	0
Propionibacterium spp.	6	1	1	3	1	0	0	0	0
Vibrio spp.	6	0	0	0	0	6	0	0	0
Shewanella spp.	6	0	0	0	0	6	0	0	0
Leuconostoc spp.	5	0	0	0	0	5	0	0	0
Carnobacterium spp.	5	0	0	0	0	5	0	0	0

Pseudomonas spp.	5	0	0	0	0	5	0	0	0
Escherichia spp.	4	2	0	1	0	1	0	0	0
Micrococcus sp	4	0	0	0	0	4	0	0	0
Debaryomyces spp.	3	0	0	0	0	3	0	0	0
Psychrobacter spp.	3	0	0	0	0	3	0	0	0
Vagococcus spp.	3	0	0	0	0	3	0	0	0
Arthrobacter spp.	2	0	0	0	0	2	0	0	0
Weixella spp.	2	0	0	0	0	1	0	0	1
Flavobacterium spp.	1	0	0	0	0	1	0	0	0
Alteromonadaceae spp.	1	0	0	0	0	1	0	0	0
Rhodopseudomonas spp.	1	0	0	0	0	1	0	0	0
Halomonas spp.	1	0	0	0	0	1	0	0	0
Megasphaera spp.	1	1	0	0	0	0	0	0	0
Methylococcus spp.	1	0	0	0	0	1	0	0	0
Phaeobacter spp.	1	0	0	0	0	1	0	0	0
Paffia spp.	1	0	0	0	0	1	0	0	0
Hanseniaspora spp.	1	0	0	0	0	1	0	0	0
Zooshikella spp.	1	0	0	0	0	1	0	0	0
Luteimonas spp.	1	0	0	0	0	1	0	0	0
Rhodococcus spp.	1	0	0	0	0	1	0	0	0
Microbacterium spp.	1	0	0	0	0	1	0	0	0
Sphingopyxis spp.	1	0	0	0	0	1	0	0	0
Leucobacter spp.	1	0	0	0	0	1	0	0	0
Dietzia spp.	1	0	0	0	0	1	0	0	0
Plesiomonas spp.	1	0	0	0	0	1	0	0	0
Hafnia spp.	1	0	0	0	0	1	0	0	0
Enterobacter spp.	1	0	0	0	0	1	0	0	0
Citrobacter spp.	1	0	0	0	0	1	0	0	0
Lysinibacillus spp.	1	0	0	0	0	1	0	0	0
Staphylococcus spp.	1	0	0	0	0	1	0	0	0
Streptomyces spp.	1	0	0	0	0	0	1	0	0
Mucor spp.	1	0	0	0	0	0	1	0	0
Pantoea spp.	1	0	0	0	0	1	0	0	0
TOTAL	921								

There were a total of 921 scientific papers assessing the benefits of probiotics on the immune system of animals (Table 6). This collection of papers represents more than 60% of documents accepted by the process of selection. The main groups of microorganisms explored in this search are *Lactobacillus spp., Bacillus spp., Enterococcus spp., Saccharomyces spp., Bifidobacterium spp., Pediococcus spp., Streptococcus spp., Aspergillus spp., Clostridium spp.,* and *Candida spp*.

3.3.2. Prebiotics

3.3.2.1 Definition of "Prebiotic"

"Prebiotics are non digestible food or feed ingredient that beneficially affects the host by selectively stimulating the growth and / or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson & Roberfroid, 1995; Roberfroid, 2007). In

order for a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial luminal / systemic effects within the host (Manning & Gibson, 2004). The aforementioned definition might be considered restrictive in that it does not accept all of the compounds introduced in the list. We included compounds such as LPS as a prebiotic (LPS was included in this section, due to the fact that its application was not used as an antigen or a challenge) (see Table 7). The main groups of prebiotics studied in this search are Mannan oligosaccharides (MOS), Glucan, Fructoologosacharides (FOS), Yeast cell wall (YCW), Inulin and Chitoologosaccharides (COS). The main action of the prebiotic at the intestinal tract is to enhance animal health status, due to its unique properties.

3.3.2.2 List of substances / agents (Prebiotics)

Table 7: Type of prebiotics studied across different animal species

Substance / Agent	Number of articles	Porcine	Poultry	Bovine	Caprine & Ovine	Fish	Rabbits	Equine	Pets
Mannan oligosaccharide (MOS)	93	12	48	2	2	25	1	0	4
Glucan	62	9	19	2	1	31	0	0	0
Fructooligosaccharide (FOS)	45	4	13	0	1	15	0	2	10
Yeast cell wall (YCW)	43	6	22	1	1	10	0	0	3
Inulin	29	7	10	1	1	6	0	0	4
Chitooligosaccharide (COS)	12	6	4	1	0	1	0	0	0
Galactooligosaccharides (GOS)	10	2	2	0	0	3	0	1	2
Lipopolysaccharide (LPS)	6	3	0	1	0	2	0	0	0
Arabinoxylan oligosaccharides (AXOS)	5	1	0	0	0	3	1	0	0
Inactivated yeast-bacteria	5	3	0	0	1	1	0	0	0
Xylooligosaccharide (XOS)	5	2	2	0	0	1	0	0	0
Galactomanan	4	4	0	0	0	0	0	0	0
Probiotic in general terms	4	0	3	0	0	1	0	0	0
Transgalactooligosaccharide (TOS)	3	0	1	0	0	2	0	0	0
Galactoglucomannan oligosaccharide-arabinoxylan complex (GGMO-AX)	3	0	3	0	0	0	0	0	0
Levan	3	1	1	0	0	1	0	0	0
Polydextrose	3	2	0	0	0	0	0	0	1
Peptidoglycan	1	0	0	0	0	1	0	0	0

Chitin	1	0	0	0	0	1	0	0	0
Galacto-mannan-oligosaccharides (GMOS)	1	1	0	0	0	0	0	0	0
Acidic oligosaccharides (AOS)	1	0	0	0	0	0	0	1	0
Arabinogalactan	1	0	0	0	0	0	0	0	1
Phosphorylated mannans (MAN)	1	1	0	0	0	0	0	0	0
Arabinoxylan	1	0	1	0	0	0	0	0	0
Mannobiose	1	0	1	0	0	0	0	0	0

TOTAL	**343**

3.3.3. Plant extracts

3.3.3.1 Definition of "Plant extract"

Plant extracts or phytogenic feed additives comprise a large number of compounds or variety of herbs, spices, and products derived thereof; essentials oils are such products. A common feature of phytobiotics is that they are a highly complex blend of bioactive components; these products are also called "Plant Secondary Metabolites" (PSM) and represent a diverse group of natural products, some of which may have nutritive values but the majority of which do not and might even be considered antinutritional (Hashemi & Davoodi, 2011).

3.3.3.2 List of substances / agents (Plant extracts)

Table 8: Type of plant extracts studied across different animal species

Substance / Agent	Number of articles	Porcine	Poultry	Bovine	Caprine & Ovine	Fish	Rabbits	Equine	Pets
Soybean derivatives	18	7	2	2	0	6	1	0	0
Thymus vulgaris derivatives	14	3	9	0	0	2	0	0	0
Allium derivatives	12	1	6	2	0	3	0	0	0
Plant extract in general-others	12	7	0	0	0	5	0	0	0
Carvacrol	11	4	6	0	0	1	0	0	0
Curcuma longa derivatives	11	2	6	2	0	1	0	0	0
Astragalus derivatives	10	3	5	0	1	1	0	0	0
Cinnamon derivatives	9	4	4	1	0	0	0	0	0
Other Chinese herbs	8	4	2	1	0	1	0	0	0
Capsicum derivatives	7	3	3	1	0	0	0	0	0
Achyranthes derivatives	6	2	1	0	0	3	0	0	0
Echinacea	6	2	3	0	0	2	0	0	0

Origanum derivatives	6	1	5	0	0	0	0	0	0
Nigella sativa derivatives	4	0	4	0	0	0	0	0	0
Beta vulgaris	4	1	0	0	0	0	1	0	2
Glycyrrhiza	4	2	1	0	0	1	0	0	0
Sugar cane	4	2	2	0	0	0	0	0	0
Chicory	3	0	1	0	0	0	1	0	1
*Plantago*derivatives	3	0	0	0	0	1	0	1	1
Citrus by product	3	1	1	0	0	1	0	0	0
Syzygium aromaticum	3	1	2	0	0	0	0	0	0
Zingiber officinale	3	0	1	0	0	2	0	0	0
Artemisia derivatives	3	1	2	0	0	0	0	0	0
Bran derivatives	3	2	1	0	0	0	0	0	0
Pinus derivatives	3	2	1	0	0	0	0	0	0
Propolis	3	0	1	0	0	2	0	0	0
Tannin	3	2	1	0	0	0	0	0	0
Cuminum cyminum	2	0	2	0	0	0	0	0	0
Medicago sativa	2	0	1	0	0	0	1	0	0
Ginkgo biloba	2	0	2	0	0	0	0	0	0
Carotenoids	2	0	2	0	0	0	0	0	0
Pisum sativum	2	1	0	0	0	0	0	0	1
Zea mays	2	1	1	0	0	0	0	0	0
Carthamus	2	0	1	0	0	1	0	0	0
Solanum tuberosum	2	1	1	0	0	0	0	0	0
Laurus nobilis	2	0	2	0	0	0	0	0	0
Humulus	2	0	2	0	0	0	0	0	0
Urtica dioica	2	0	0	0	0	2	0	0	0
Linum usitatissimum	2	1	0	0	0	1	0	0	0
Camellia sinensis	2	0	1	1	0	0	0	0	0
Magnifera indica	2	0	0	0	0	2	0	0	0
Prunus derivatives	2	0	2	0	0	0	0	0	0
Mentha piperita	2	0	1	0	0	1	0	0	0
Oscimum sanctum	2	0	0	0	0	2	0	0	0
Panax ginseng	2	0	1	0	0	1	0	0	0
Rubus coreanus	2	0	1	0	0	1	0	0	0
Sanguinaria canadensis	2	0	1	0	0	1	0	0	0
Trigonella derivatives	2	1	0	0	0	1	0	0	0
Yucca Schidigera	2	0	2	0	0	0	0	0	0
Uncaria tomentosa	2	0	0	0	0	2	0	0	0
Achillea	1	0	1	0	0	0	0	0	0
Agrimonia	1	1	0	0	0	0	0	0	0
Alnus firma	1	0	0	0	0	1	0	0	0
Anethole	1	0	1	0	0	0	0	0	0

Larix	1	0	0	0	0	0	0	0	1
Cyamopsis derivatives	1	0	0	0	0	0	0	0	1
Canavalia ensiformis	1	0	1	0	0	0	0	0	0
Cucurbita	1	0	0	0	0	1	0	0	0
Lonicera	1	0	0	0	0	1	0	0	0
Myrtus communis	1	0	1	0	0	0	0	0	0
Foeniculum vulgare	1	0	1	0	0	0	0	0	0
Pimpinella anisum	1	1	0	0	0	0	0	0	0
Juniperus communis	1	0	0	1	0	0	0	0	0
Viscum album	1	0	0	0	0	1	0	0	0
Gossypium	1	0	1	0	0	0	0	0	0
Malus domestica	1	0	0	0	0	0	1	0	0
Avena sativa	1	0	0	0	0	0	1	0	0
Isoflavones	1	0	1	0	0	0	0	0	0
Helianthus tuberosus	1	1	0	0	0	0	0	0	0
Kalopanax pictus	1	0	0	0	0	1	0	0	0
Lactuca indica	1	0	0	0	0	1	0	0	0
Lupinus perennis	1	0	0	0	0	1	0	0	0
Garcinia mangostana	1	0	0	0	0	1	0	0	0
Cocos nucifera	1	0	1	0	0	0	0	0	0
Silybum marianum	1	0	1	0	0	0	0	0	0
Aconitum koreanum	1	0	0	0	0	1	0	0	0
Phoenix dactylifera	1	0	0	0	0	1	0	0	0
Eleutherococcus senticosus	1	0	0	0	0	0	1	0	0
Piper nigrum	1	0	1	0	0	0	0	0	0
Coriandrum sativum	1	0	1	0	0	0	0	0	0
Epimedium derivatives	1	0	1	0	0	0	0	0	0
Houttuynia cordata	1	0	1	0	0	0	0	0	0
Quillaja saponaria	1	1	0	0	0	0	0	0	0
Acacia derivatives	1	0	1	0	0	0	0	0	0
Arthropodium cirratum	1	0	1	0	0	0	0	0	0
Rosmarinus officinalis	1	0	0	0	0	1	0	0	0
Azadirachta indica	1	0	0	0	0	1	0	0	0
Ceratonia siliqua locust bean	1	1	0	0	0	0	0	0	0
Ilex paraguarensis	1	0	1	0	0	0	0	0	0
Withania somnifera	1	0	0	0	0	1	0	0	0
Broussonetia kazinoki	1	0	0	0	0	1	0	0	0
Raw fibre	1	0	0	0	0	1	0	0	0

TOTAL	**263**

The bibliographic revision has detected 263 papers in which the application of plant extracts in animal feeding was assessed (Table 8). The main compounds studied are: soybean derivatives, *Thymus vulgaris* derivatives, *Allium* derivatives, Carvacrol, *Cucuma longa* derivatives, *Astralugus* derivatives, *Cinnamon* derivatives, *Capsicum* derivatives, *Achyranthes* derivatives, *Echinacia*, *Origanum* derivatives. A number of papers explored the effects of blend compounds.

3.3.4. Animal by-product

3.3.4.1 Definition of "Animal by-product"

Animal by-products are entire bodies or parts of animals, products of animal origin, or other products obtained from animals; these are not intended for human consumption, and include oocytes, embryos, and semen. Feeding animals with these products may improve the animal's performance and/or may modulate their immune system.

3.3.4.2 List of substances / agents (Animal by-product)

Table 9: Type of animal by-product studied across different animal species

Substance / Agent	Number of articles	Porcine	Poultry	Bovine	Caprine & Ovine	Fish	Rabbits	Equine	Pets
Lactoferrin	15	5	3	2	1	3	0	0	1
SDP	11	11	0	0	0	0	0	0	0
Antibodies	11	7	1	2	0	1	0	0	0
Other	10	3	4	0	0	3	0	0	0
Oils	4	2	2	0	0	0	0	0	0

TOTAL	**51**

The bibliographic revision has detected 51 papers in which the application of animal by-product in animal feeding was assessed (Table 9). The main compounds studied include: lactoferrin (LF), spray-dried products (SDP), and antibodies.

3.3.5. Other substances

3.3.5.1 Definition of "Other substances"

This section includes a group of compounds also used as feed additives, such as technological, nutritional, or zootechnical feed additives. The section also includes substances present in natural products, such as algae or fungi mushroom.

3.3.5.2 List of substances / agents (Other substances)

Table 10: Type of other substances / agents studied in different animal species

Substance / Agent	Number of articles	Porcine	Poultry	Bovine	Caprine & Ovine	Fish	Rabbits	Equine	Pets
Organic acids derivatives / Fatty acids	51	14	31	1	0	4	1	0	0
Vitamins	42	3	13	4	1	20	0	0	1
Minerals	39	11	12	6	2	6	0	2	0
Amino acids and derivatives	38	19	12	0	0	6	1	0	0
Others	32	4	15	4	0	7	0	0	2
Algae	27	6	3	1	0	17	0	0	0
Enzymes	12	0	9	0	1	2	0	0	0
Fungi - Mushroom	11	0	10	0	0	1	0	0	0
Peptides	10	3	5	0	0	2	0	0	0
Nucleotides	9	4	1	1	0	3	0	0	0
Antibiotic	9	1	8	0	0	0	0	0	0
Lactulose	7	3	0	4	0	0	0	0	0

TOTAL	**287**

The bibliographic revision detected 287 papers in which the application of other substances / agents in animal feeding was assessed (Table 10). The main compounds studied were: Organic acids derivatives / fatty acids, vitamins, minerals, amino acids, and derivatives, *algae*, enzymes, etc.

3.4. Prevalent mode of action of substances / agents

This section describes the mode of action for major substances, by target species. We have considered the prevalent mode of action, and a summary of this mode of action is described in the list of articles included.

3.4.1. Porcine

3.4.1.1 Porcine – probiotics

The most common additives studied in swine, with regard to its effects on immunity, were probiotics. Among these (186 studies), most were regarded as *Lactobacilli* (69 studies), *Bacillus* (31 studies), *Enterococcus* (29 studies), and *Saccharomyces* (30 studies) (Table 11).

Table 11: Mode of action of different probiotics in porcine

Porcine - Probiotic	Number of articles	Mode of action
Lactobacillus spp.	**69** Refs: 9177 2496 1402 6395 5094 3751 12889 12893 4135 3509 1901 6149 3580 5185 3579 6718 1549 3428 12200 4401 4496 1874 5888 2333 4314 5743 12136 6978 4562	The function of *Lactobacillus* is to keep the gut micropopulation in a balanced state and to prevent the proliferation of pathogenic microorganisms (increasing lactic acid and total volatile fatty acids (VFA) concentrations). *Lactobacillus* enhanced the level of mucosal proteins involved in energy metabolism, cell structure, and mobility in the jejunum of weaning piglets. The higher VFA concentration in the colon of the lactic acid bacteria (LAB)-fed piglets might have contributed to the lower rates of diarrhoea. The higher concentrations of lactic and acetic acid in the ileum and colon of LAB-fed piglets could have been the result of the higher numbers of LAB in these animals, as LAB during fermentation may either produce lactic acid alone or may produce both lactic and acetic acid. The LAB numbers and their major fermentation products may indirectly affect the concentrations of acetic, propionic and butyric acid. Reduction of pathogenic microorganisms in the gut of animals can be explained by the fact that LAB reduces pH in the luminal contents, which can inhibit the proliferation of pathogenic bacteria. Villous heights (VH) of the small intestine were increased by administration of *Lactobacillus*. The reduced lymphocyte infiltration in the gastrointestinal tract in treated animals may indicate reduced inflammation. The duodenum colonisation with *Lactobacillus* can contribute to a variety of mucosal immune responses, including an increase in the expression of TLR2, TLR9, the nucleotide-binding oligomerization domain (NOD)-1, and the secretion of cytokines; this also elevates the number of immunoglobulin (Ig)-A producing cells. *Lactobacillus* increases the amount of intraepithelial lymphocytes (IELs) and IgA-producing cells in the intestinal tract, resulting in the development of intestinal mucosal immunity, whereas the effect on the secretion of interleukin (IL)-6 may also provide beneficial assistance for the development of intestinal mucosal immunity. IL-6 was elevated and regulated in *Lactobacillus*-stimulated cells. TLR2 might contribute to the induction of cytokines mediated by *Lactobacillus*. TLR2 has an important role in anti-

	10989 12122 3879 3937 8522 1916 11382 8281 6105 3510 7322 12762 11167 11055 12165 1674 10634 11708 6278 13004 5655 12723 1873 12899 6811 3581 1783 2001 5410 699 11429 5574 1785 1777 12076 11976 10477 12724 12164 2486	inflammatory activity through the modulation of A20 and B-cell lymphoma (Bcl)-3, but not of the Mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1). *Lactobacillus* appears to have similar effects and strongly up-regulated A20, BCL-3. The toll interacting protein (Tollip), Single-immunoglobulin interleukin-1 receptor-related (SIGIRR), and MKP-1 levels in porcine intestinal epitheliocytes (PIE) cells also had similar effects. Levels of Bcl-3, SIGIRR, and Tollip were higher in PIE cells treated with the *Lactobacillus* strain, indicating that other pattern recognition receptors (PRRs) could be involved along side the TLR2 in the upregulation of these TLR negative regulators. Il-1β, Il-8, Il-17 and TNF-α, pro-inflammatory cytokines were observed to have lower mRNA expression after *Lactobacillus* administration. The results indicate that the NOD-like receptor family, pyrin domain containing 3 (NLRP3), expression is upregulated by TLR2, TLR9, NOD1 and NOD2 agonists in adult and new-born porcine Gut-Associated Lymphoid Tissue (GALT), where these receptors can respond to intestinal bacterial components. The present findings suggest that immunobiotic *Lactobacillus* strains directly promote NLRP3 expression via TLR and NOD-mediated signalling, resulting in the induction of appropriate NLRP3 activation in porcine GALT. The interaction of the *Lactobacillus* strain with Intestinal Epithelial Cells (IECs) would induce the upregulation of MKP-1, Bcl3 and A20 expression. *Lactobacillus* could be taken by Antigen Presenting Cells (APCs) indirectly through M cell transport or by direct sampling from the intestinal lumen, inducing an increase in the production of the immunoregulatory cytokines IL-10 and the transforming growth factor (TGF)-β by CD172a+ CD11R1− and CD172a+ CD11R1high cells as well as the expression of SIGIRR, IRAK-M and A20. In addition, through its direct interaction with CD172a− CD11R1 low cells, the TL2937 strain would have the capacity to improve Th1 responses by increasing the production of interferon (IFN)-γ. *Lactobacillus* would be able to stimulate the production of immunoregulatory factors such as TGF-β in IECs, which would increase the expression of Bcl-3 and the production of IL-10 in CD172a+ APCs. Then, *Lactobacillus* would functionally modulate IECs and APCs in order to improve resistance to infections and to avoid non-protective inflammation. Oral administration of *Lactobacillus* indicated an increased percentage of peripheral blood CD4+ T lymphocytes. *Lactobacillus* seemed to stimulate T-cell differentiation, which could play a role in the regulation of immune function. Oral administration of *Lactobacillus* increased ileum interferon-γ and Tumour Necrosis Factor (TNF)-α expression, which indicated that *Lactobacillus* could improve mucosal immune activity. Decreased CD25 induction on the surface of CD4+ (helper) T cells and monocytes in probiotic-fed pigs, compared with control animals, may suggest that these cells are less active in response to a challenge following probiotic administration, which in turn suggests a potential immunomodulatory role of the five-strain probiotic. The decreased surface expression of Cytotoxic T-Lymphocyte Antigen (CTLA)-4 on CD4+(helper) T cells following stimulation, which was observed as a result of probiotic treatment, is indicative of altered regulation of the inflammatory T-cell response. The increase in the CD4+CD8+ double positive T-cell subset observed after probiotic administration may reflect an increase in memory T cells in response to any subclinical infection to which animals were exposed.
Bacillus spp.	**31** Refs: 101 10149 10132 10566	The colonisation of *Bacillus* can contribute to a variety of mucosal immune responses, including increased expression of TLR-2 and the secretion of cytokines; they can also elevate the number of IgA producing cells. The administration of the *Bacillus* mainly stimulated the gene expression of IL-6, porcine β-defensin (pBD)-2, and TLR2 in the intestinal tract. Under the influence of *Bacillus*, the population of intraepithelial CD8+ T cells was significantly enhanced. Feed supplementation of

	2177 2496 809 33 5757 34 3580 4949 3971 6138 4556 1301 12123 1874 10634 5872 1873 5732 9003 3898 3581 1783 5410 5574 1785 10146 10130	*Bacillus* to sows and piglets was shown to affect the intestinal immune system of the piglets at the time of weaning (age 28 days) and shortly thereafter. The intestinal epithelial CD8/CD3 double-positive cell populations were enhanced in the probiotic group. Therefore, the increase in Lamina propria CD25+ cells is indicative of immune stimulation. The ratio of IEL (CD45+) to enterocytes was lower in the *Bacillus* treated group, which could be explained by lower frequencies of CD8 γδ+ T cells in the jejunal epithelium. The relative number of memory helper cells was diminished in the probiotic group. The population of bright CD8+ T cells (CD8high/CD3+), generally considered cytotoxic T-cells, was lower. Perforin was absent from most αβ T cells and also absent from γδ T cells in porcine blood. A reduction of relative numbers of γδ T cells in the blood and in the jejunal epithelium of the piglets of the group fed with Bacillus was observed. The elevated CD4+:CD8+ ratio in the *Bacillus* group might have resulted from continuous feeding of *Bacillus* and the subsequent prolonged presence of *Bacillus* antigens, which might be equivalent to a secondary immune response against infection. The increased concentrations of serum immunoglobulins IgG and IgA observed for pigs fed diets supplemented with *Bacillus* fermentation biomass is reflective of improved immune function. *Bacillus* has the potential for increasing serum antibody levels and the consequent effects on weaning stress and health status may have contributed to the improved growth performance exhibited by pigs fed the supplemented diets. Changes in small intestinal morphology, and in particular an increase in villus height and VH:Crypt depth (CD) ratio, were found in pigs fed diets supplemented with the *B. subtilis* fermentation biomass; these could be indicative of improved gut health and digestive capacity. Other mechanisms through which *Bacillus* strains may alter the type of microflora in the gastrointestinal tract could be the decreased oxidation–reduction potential caused by the germination of spores in the intestine, which has been shown to benefit the growth of lactobacilli. In addition, *Bacillus* strains could produce metabolites that inhibit pathogens, as some *Bacillus* species used in commercially available products have the ability to produce antimicrobials such as aminocumaim A and bacteriocin. Bacillus treatment may inhibit Enterotoxigenic *Escherichia coli* (ETEC)-induced pro-inflammatory responses by suppression of MAP-kinase (MAPK) signalling pathways in intestinal epithelial cells, which provides a rationale for decreased incidence of diarrhoea in the *Bacillus*-supplemented diet of weaned piglets.
Saccharomyces spp.	**30** Refs: 12280 10429 2321 1901 6149 3580 3428 1874 1873 449 7464 11756 8700 6840 2013 6038	*Saccharomyces cerevisiae* has been shown to inhibit pro-inflammatory gene expression. This inhibition is associated with the modulation of both extracellular signal-regulated kinase (ERK)-1/2 and p38 signalling pathways, the increase of Peroxisome proliferator-activated receptor (PPAR)-c transcript expression, and the ETEC agglutination by yeasts. Live yeast reduced the expression of TLR2 and 4 in the intestine, suggesting that yeast may interfere with the *E. coli* binding to the intestinal surface. *S. cerevisiae* reduced ETEC F4 attachment to the ileal mucosa, and it seems likely that *S. cerevisiae* reduced ETEC F4 attachment to the ileal mucosa through other mechanisms, such as competition, modulation of intestinal bacterial populations, and increased barrier function. The serum levels of gamma-globulins, lysozyme, IFN-γ, IL-1b, and IL-6 levels increased after *Saccharomyces* administration. These observations may indicate activation of the immune system or an inflammatory reaction induced by an infectious agent. The addition of *Saccharomyces* down-regulate ETEC-induced gene expression of TNF-α, Granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6, chemokine (C-C motif) ligand (CCL)-2, chemokine (C-X-C motif) ligand (CXCL)-2 and CCL20, reducing the overall proinflammatory

	7370 12686 10430 5358 11673 3581 5410 5574 1785 451 448 12829 12827 12831	state caused by ETEC. Yeast might change the immune function of the gut by triggering a Th-1 response. A possible explanation may be that yeast triggers gut epithelium or gut-associated lymphoid tissue Th-1 responses by activating macrophages. IFN-γ, which can activate phagocytosis by macrophages, was increased in gut mucosa by yeast supplementation. Thus, weaning-induced systemic immune response alleviated by yeast cells may be due to improvements in Th-1 response in gut mucosa. Yeast has been shown to promote the growth of lactobacilli, suggesting that the lower numbers of faecal coliforms in the treated group could be interpreted as a positive effect of yeast on the enteric ecosystem, intestinal integrity, and health. The higher proportions of CD4+, CD8+ and CD4+CD8+ peripheral blood lymphocytes in the yeast group suggest that systemic cell-mediated immunity was activated by the changes induced by yeast. In pigs, CD4+ and CD4+CD8+ lymphocytes are T-helper cells. Activation of these subpopulations stimulates plasma cells for antibody production, and changes in these subpopulations might indicate a concurrent increase in plasma antibodies. No changes in gut architecture, mucus thickness, goblet cell population, or cortisol level were observed.
Enterococcus spp.	**29** Refs: 6683 9177 10182 6889 8598 10566 11701 4588 6274 10534 3580 5738 1235 6757 5736 4695 661 11003 12123 3579 6718 4401 6811 3581 945 1784 946 5737 10131	Findings suggest that *E. faecium* EF1 possesses remarkable immunomodulatory activity in intestinal mucosa by suppressing synthesis of the proinflammatory cytokines (IL-1β, IL-6, IL-12, IL-8 and IFN-γ). IL-10 was statistically increased in both jejunal and ileal mucosa of probiotic supplemented piglets. TNF-α production suggests that *E. faecium* EF1 may promote early activation of the intestinal immune system. TLR2 and TLR9 transcription levels, measured by quantitative real time (qRT)-polymerase chain reaction (PCR), were up-regulated in the jejunal mucosa of piglets treated with *E. faecium* EF1. Compounds of *E. faecium* EF1 cell-wall could act as adjuvants of the mucosal immune response and *E. faecium* EF1 Deoxyribonucleic acid (DNA) is major component of the *E. faecium*-mediated activation. Application of *E. faecium* affects the systemic and intestinal immunity of weaning piglets by diminishing the population of circulating and intraepithelial CD8αβ T cell. The levels of cytotoxic T cells (CD8+) in the jejunal epithelium of piglets in the probiotic group were significantly reduced. The observed decline in the CD8+ IEL populations was the result of an increased population of CD4-CD8 cells, i.e. that the *E. faecium* probiotic strain had a proliferative effect on a cell population of unknown function. The reduction in β-haemolytic *E. coli* isolates in probiotic-treated animals suggests a reduced pathogenic load in the probiotic group. Piglets supplemented with *E. faecium* NCIMB 10415 exhibited a post-weaning dysregulation in the expression patterns of both pro- and anti-inflammatory cytokine expression in the intestinal tissues and spleen. Piglets in the supplemented group showed significantly reduced levels of IL-8, IL-10 and the co-stimulatory molecule CD86 mRNA expression in ileal Peyer's patches. The expression of CTLA4, an inhibitor of T-cell activation/proliferation, showed similar levels of expression in all tissues examined, particularly in ileal Peyer's patches post-weaning, where IL-8, IL-10 and CD86 transcript levels were significantly reduced relative to control animals. Blood serum cytokine protein levels showed elevated TGF in pre-weaning piglets which, together with IL-6, may have suppressed IFN production in the probiotic-fed animals. These observations suggest that prolonged feed supplementation of sows and piglets with the probiotic strain *E. faecium* NCIMB 10415 (SF68) results in an anti-inflammatory/immuno-suppressive response in piglets.
Pediococcus spp.	**11**	The attachment of ETEC F4 to the ileal mucosa was significantly lower for

	Refs: 2321 3579 1549 4401 12122 11708 6201 6038 9003 9004 3581	the pigs receiving *P. acidilactici* + *S. cerevisiae*. Ingestion of *Pedicoccus* after birth has the potential to modulate the establishment of CD8+ cells and the secretion of intestinal IgA in the ileum. IL-6 and IL-8 were significantly increased in pigs receiving *P. acidilactici* + *S. cerevisiae*. The expression of pBD-2, (antimicrobial peptide) and of IL-12p35 (activation of mucosal innate immunity against enteric pathogens) also tended to be increased in the *P.acidilactici* group only. The CD8+low T lymphocytes in the ileum and CD8+high cells in the mesenteric lymph nodes (MLN) tended to be increased following administration of *P. acidilactici* compared with the control group. The results indicate that the increased number of lymphocyte subpopulations in the gut could result from stimulation of the local immune system by probiotic bacteria. After challenge with ETEC, the bacterial translocation to MLN was reduced. Enterocyte proliferation in crypt epithelium decreased after the administration of *Pedicoccus*. Villus length, crypt depth, mucus-producing cell counts, and thickness of the mucus layer remained unaffected in the small intestine.
Bifidobacterium spp.	**7** Refs: 9177 4135 11516 6718 11443 2001 3329	*Bifidobacteria longum* BB536 and *B. breve* M-16V strains significantly downregulated levels of IL-8, monocyte chemotactic protein (MCP)-1 and, IL-6 in PIE cells challenged with heat-killed enterotoxigenic *Escherichia coli*. Moreover, BB536 and M-16V strains attenuated the proinflammatory response by modulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and MAPK pathways. In addition, the observations provide evidence for a key role for the ubiquitin-editing enzyme A20 in the anti-inflammatory effect of immunobiotic bifidobacteria in PIE cells. Immunoregulatory strains interacted with TLR2, upregulated the expression of A20 in PIE cells, and beneficially modulated the subsequent TLR4 activation by reducing the activation of MAPK and NF-κB pathways and the production of proinflammatory cytokines. A linear effect of the dose of *B. animalis* on the expression of the TLR2- encoding gene in the ileocaecal MLNs was identified.
Aspergillus spp.	**5** Refs: 1901 4320 6149 1874 1873	*Aspergillus oryzae* increased TLR4, IL-8, and TNF-α expression when we incubated porcine intestinal epithelial cells (IPEC)-J2 with *A. oryzae* in the absence of *E. coli*. These results suggest that the *A. oryzae* likely presents some immunogenic compound that interacts with enterocytes and triggers inflammatory response. Itis possible that this effect is due to galactomannans present in the membrane of the *Aspergillus*.
Escherichia spp.	**2** Refs: 808 1328	The improved performance observed with probiotic (2 *E. coli* strains) supplementation is likely mediated by a reduction in *E. coli* K88 colonisation of the mucosal surface and subsequent reduction of post-weaning diarrhoea. *Escherichia coli* Nissle 1917 provided a significant favourable trophic effect on the colonic mucosa. In the jejunum, *E. coli* administration was associated with significantly lower values of height of cryptal mucosa as well as height and width of villi. In the colon, the height of cryptal mucosa was significantly higher in the *E. coli* group.
Streptococcus spp.	**1** Refs: 4135	In porcine diets, *Streptococcus* is supplemented as a mixture with other probiotics. Therefore, its specific mode of action could not be identified.
Propionibacterium spp.	**1** Refs: 2121	Colonic mucosa explants of pigs treated with *Propinobacterium* secreted less IL-8 and TNF-α, either in basal conditions or after a lipopolysaccharide (LPS) challenge. By contrast, the gut structure, barrier function, microbial diversity, and colonic short-chain fatty acid content remained unchanged, assuming maintenance of normal intestinal

		physiology.
Megasphaera spp.	**1** Refs: 12762	The addition of *M. elsdenii* to diets supplemented with *L. plantarum* Lq80 (LM) may increase butyrate production. The caecal and colonic crypt depths were longer in piglets in the LM group than in those in the *L. plantarum* and control groups. The colonic short chain fatty acid (SCFA) level and the thickness of the colonic mucosa were significantly higher in piglets in the LM group.

3.4.1.2 Porcine – prebiotics

Sixty-four studies concern prebiotic, the most common of which were mannan oligosaccharides (MOS, 12 studies), glucans (9 studies), fructooligosaccharides (FOS, four studies), inulin (source of FOS, seven studies), the yeast cell wall (YCW), (source of MOS among other substances, six studies) and chitooligosaccharides (COS, six studies).

Table 12: Mode of action in different prebiotics in porcine

Porcine - Prebiotic	Number of articles	Mode of action
Mannan oligosaccharide (MOS)	**12** Refs: 6033 1720 4320 6149 1718 1719 1570 1901 1337 8524 9004 2207	MOS reduced IL-1, IL-8, and TNF-α expression and showed some effects in TLR4 and TLR5 expression as well as an increase in IgG concentration. Gene expression of IL-1α, IL-6, myeloid differentiation factor 88, TLR 4, major histocompatibility complex (MHC) II, and dead box polypeptide increased in peripheral blood mononuclear cells (PBMC) of MOS-fed pigs. This suggests that MOS enhances disease resistance in pigs and supports the fact that MOS induced a rapid increase in leukocytes following infection. In PRRS infected pigs, MOS reduced the expression of IL-1 β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, and TLR4 genes in PBMC. MOS decreased the jejunal numbers of enterobacteria and increased the villus:crypt ratio. In other studies, dietary MOS did not affect the serum concentration of TNF-α and tended to increase that of IL-10.
Glucan	**9** Refs: 4671 5712 2982 4393 10969 12229 12118 10748 11086	β-Glucan did not alter final BW, ADG, or G:F. The β-glucan content of the diet did not impact villus height or crypt depth. Plasma IL-10 levels were also increased in the piglets fed with β-glucan whereas plasma IFN-γ and TNF-α levels were decreased. Treatment of β-glucan alone might decrease inflammatory response against Gram-negative bacterial infection via the inhibition of pro-inflammatory cytokine production and the enhancement of anti-inflammatory cytokine production. Supplementation of dietary β-glucan resulted in an increased concentration of peripheral erythrocytes, leukocytes, lymphocytes, and eosinophils. No obvious dose-effects were observed except in the concentration of CD4+ cells, which increased as the β-glucans content increased. Blood immune cell variables remained within physiological ranges, supporting the clinical observation that supplementing the diet of pigs with β-glucans did not adversely affect their health. The proportion of CD45RA+ T-cell populations (mesenteric lymph nodes and Peyer's patch CD4+ cells and Peripheral blood lymphocyte (PBL) CD8+ cells) was greater in pigs fed β-glucan.

		β-Glucans did not stimulate any pro- or anti-inflammatory cytokine markers in the ileal epithelial cells. In contrast, the expression of a panel of pro- and anti-inflammatory cytokines (IL-1a, IL-10, TNF-α and IL-17a) was down-regulated in the colon following exposure to β-glucans. Piglets receiving food supplemented with β-glucans for two weeks after weaning showed a decreased susceptibility to ETEC. Oral supplementation with β-1,3/1,6-glucan resulted in temporarily increased serum IgG concentrations in weanling piglets.
Inulin	**7** Refs: 6718 3852 7484 11186 11251 8519 945	The overall performance of piglets was unaffected by treatment. Inulin increased total aerobes in the stomach and jejunum, whereas enterococci declined in the colons of the inulin group. Furthermore, decreasing colonic acetic acid and increasing lactic acid was observed for inulin. Inulin may contribute to the regulation of Fe absorption either directly by affecting SCFA synthesis or through changes in bacterial population and/or by affecting mucin gene expression. Inulin is able to increase the level of cytokine (IL-1β, IL-8, IL-6, TNF-α, IFN-γ) and IgA concentration.
Yeast cell wall (YCW)	**6** Refs: 10077 11756 2859 4555 12076 7390	YCW β-glucan increased body weight (BW) and average daily gain (ADG). YCW increased TNF-α expression in the spleen and IL-1Ra (receptor antagonist) in the intestine. Red blood cells (RBC), haemoglobin, haematocrit (Hct) value, mean cell volume, mean cell haemoglobin, percentage of lymphocytes in the leukocyte population, villus length, and crypt depth were greater. Eosinophils in the leukocyte population tended to be greater and concentrations of neutrophils in the leukocyte population and percentages of CD4 and CD8 cells were lower. The CD4:CD8 ratio tended to be lower as well. Yeast-derived protein seemed to enhance IgG and IgA levels.
Chitooligosaccharide (COS)	**6** Refs: 12708 5642 6409 12117 12709 12728	After chitosan adheres to the intestinal mucosa, its amine is recognised by the immune system. Immune response pathways are activated, and the gut-associated lymphoid immune system is stimulated to produce lymphokines and inflammatory mediators, secrete cytokines IL-1, IL-6, etc., reduce calprotectin and TLR4 protein expression, and enhance the cell-mediated immune response. COS helps to prevent inflammatory intestinal disorders, including weaning-associated intestinal inflammation. COS increases some presumably-beneficial bacterial species and SCFA concentrations in the intestinal luminal content while decreasing the amounts of several potential pathogens, concentrations of ammonia, and branched-chain fatty acid (BCFAs) in this content. COS decreased feed conversion ratio, villus width, crypt depth, and TLR4 mRNA expression but increased villus length, villus length/crypt depth, and GCs. COS enhanced IL-1β gene expression in jejunal mucosa and lymph nodes as well as serum levels of IL-1β, IL-2, IL-6, IgA, IgG and IgM. COS may enhance the cell-mediated immune response in piglets that are weaned early by modulating the production of cytokines and antibodies.
Galactomannan	**4** Refs: 451 448 450 449	Addition of Galactomannan down-regulate ETEC-induced gene expression of TNFα, GM-CSF, IL6, CCL2, CXCL2 and CCL20, reducing the overall proinflammatory state caused by ETEC. GM inhibited the association of *Salmonella* with intestinal epithelial cells (IECs) in vitro. The addition of Galactomannan to dendritic cells (DCs) cocultured with *Salmonella* showed higher gene expression (mRNA) for TNF-α and GM-CSF compared to that of the control with *Salmonella*. β-Galactomannan and Mannan SC reduced the secretion of CXCL8 and IL-6 induced by *Salmonella typhimurium* infection in intestinal epithelial

		cells (IPI-2I).
Fructooligosaccharide (FOS)	**4** Refs: 12188 1301 4923 7484	Higher villus density in the duodenum of piglets fed a diet with FOS.
Lipopolysaccharide (LPS)	**3** Refs: 1339 8258 2516	Piglets fed diets rich in 18:3 n-3 exhibited greater jejunal permeability to FITC-LPS. Cytokines TNF-α, IL-8 and IL-18 showed a significant increase in their levels of expression in pigs fed diets supplemented with LPS. LPS does not induce increased TLR mRNA levels in epithelial cells or in mononuclear phagocyte cells.
Galactooligosaccharides (GOS)	**2** Refs: 11568 10666	Galactooligosaccharides increase concentrations of gut beneficial bacteria, bifidobacteria and lactobacilli, as well as concentrations of short-chain fatty acids, but they decrease nutrient digestibility. Dietary GOS suppressed eosinophil infiltration in the small intestine and tended to improve the growth performance of weaning piglets.
Xylooligosaccharide (XOS)	**2** Refs: 7464 6840	The positive effect of XOS on villi length (villus height was increased) can improve the nutrient uptake from the intestine, which could result in improved growth performance. XOS seem to have a potential prebiotic effect on lactobacilli from the piglets' intestinal tracts but no effect was noted on *Bifidobacteria*.
Polydextrose	**2** Refs: 3076 12188	Concentrations of SCFA and biogenic amines increased in the large intestine. In contrast, concentrations of luminal IgA decreased distally but the expression of mucosal Cyclooxygenase (COX)-2 had a tendency to increase in the mucosa toward the distal colon. There was a reduction in concentrations of SCFA and tryptamine and an increase in concentrations of spermidine in the colon upon polydextrose supplementation. The addition of short chain FOS (scFOS) and polydextrose to formula increased acetate concentrations and decreased luminal pH on d 7 in the ascending colon. This finding may be understoodas beneficial, as an in vitro study showed that acetate inhibited the growth of many common pathogens in a concentration-dependent manner and that inhibition was most pronounced at a lower pH.
Arabinoxylan oligosaccharides (AXOS)	**1** Refs: 7758	AXOS may exert its attenuating effect on the ETEC-induced small intestinal response by influencing the expression of particular innate response proteins, such as the Prostatic Acid Phosphatase protein.
Levan	**1** Refs: 6244	Dietary supplementation with 0.10% levan type fructan improved growth performance, digestibility of nitrogen, gross energy, and faecal *Lactobacillus* counts and had a beneficial effect on immune response during an inflammatory challenge (increased white blood cells, lymphocyte percentage, serum cortisol, TNF-α, and IL-6).
Galacto-mannan-oligosaccharides (GMOS)	**1** Refs: 12728	Serum levels of IL-1β, IL-2, and IL-6 in piglets fed the GMOS diet were higher than the levels in control piglets. These findings suggest that GMOS may enhance the cell-mediated immune response in piglets weaned early by modulating the production of cytokines and antibodies.
Phosphorylated mannans (MAN)	**1** Refs: 2334	Supplementation of mannans in the diets of weanling pigs improved gain and efficiency and intermittently affected selected components of the piglets' immune function both systemically and enterically. The percentage of neutrophils was lower and the percentage of lymphocytes was higher in the blood. Lamina propria macrophages, isolated from pigs fed diets containing mannans, phagocytised a greater number of sheep red blood cells.

3.4.1.3 Porcine – plant extracts

A substantial number of studies (68) concerning plant extracts exist.

Table 13: Mode of action of different plant extracts in porcine

Porcine - Plant extract	Number of articles	Mode of action
Soybean derivatives	**7** Refs: 12980 12772 3510 1717 2744 3259 2742	Soybean oligosaccharides promoted the growth of bacterial species that are potentially beneficial to the host and increased the concentration of SCFA in the ileum and colon while simultaneously reducing the numbers of potentially harmful bacteria and the production of protein-derived catabolites. Soybean oligosaccharides was found to increase the expression of the tight junction protein ZO-1 mRNA but to diminish the expression of pro-inflamatory cytokines TNF-α, IL-1β, and IL-8 mRNA in both the ileum and colon, whereas supplementation increased that of the anti-inflammatory cytokine IL-10. SOY (soy di- and tripeptide diet) piglets had a lower crypt depth than and a similar muscle thickness to those in the negative control group. SOY peptide treated pigs had reduced IL17a and RORC gene expression compared to positive controls, suggesting that soy peptides may modulate this pathway. Cytokine IL17a activates the NF-κB and MAPK proinflammatory cascade, and recent studies indicate that RORC is necessary for T- helper (Th)-17 commitment and differentiation. Soy peptide supplementation lowered the expression of IFN-γ, TNF, and IL12b. The Treg transcription factor, FOXP3, was highly expressed in the ileum of SOY pigs. FOXP3+ Treg (the Treg lineage-specific transcription factor that suppresses RAR-related orphan receptor C (RORC) and inhibits Th17 differentiation) was upregulated. The density of cells was approximately five times more numerous in the villus Lamina Propria (LP) of the duodenal mucosa of heat-treated soy protein (HTSP) piglets than in the ethanol-treated soy protein (ETSP) group. An approximately three-fold increase in IgM and IgA, and a six fold increase in IgG1 and IgG2 positive cells,was observed in the lamina propria (LP) of the duodenal mucosa of HTSP piglets compared with ETSP piglets. The proportions of T-lymphocyte in the duodenal mucosa differed between groups with regard to CD2+ lymphocytes and especially in CD4+ T cells, which increased approximately six-fold in the epithelium and three-fold in the LP of the HTSP piglets. Beta-sitosterol (phytosterol of soybean) increased viable peripheral blood mononuclear cell (PBMC) numbers, activating swine dendritic cells (DCs). Antigenic soybean product increased diarrhoea, reducing the size of duodenal villi and eosinophil density in the duodenal mucosa, which was greater than the non-antigenic soybean product.
Carvacrol	**4** Refs: 4724 3271 7799 7484	In vitro, sub-inhibitory concentrations of carvacrol affect virulence of *Salmonella*, leading to reduced motility and the invasion of epithelial cells. A mixture composed of carvacrol, capsicum oleoresin, and cinnamaldehyde showed antioxidative properties under dietary-induced oxidative stress conditions. The mixture effectively protected the pigs' blood lymphocytes from oxidative DNA damage. Dietary XT (5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin) reduced intraepithelial lymphocyte numbers in jejunum and the percentage of blood cytotoxic cells and B lymphocytes in LN; however, XT increased blood monocytes and the density of lamina propria lymphocytes in the colon. Monoterpene phenols, such as thymol and carvacrol, interact with the cell membrane by H bonding, rendering the membranes and mitochondria more permeable, disintegrating the outer cell membrane, and inhibiting the growth of Gram (−) bacteria.
Cinnamon derivatives	**4** Refs: 6258 6269 3271	Essential oils (Thymol + cinnamaldehyde) reduced the occurrence of diarrhoea and decreased *E. coli* counts in faeces. Feeding EO increased lymphocyte transformation and leucocyte phagocytosis rates as well as the levels of IgA, IgM, C3, and C4 in blood. The addition of EO improved daily weight gain and the feed conversion ratio (FCR) of weaned pigs. Phenylpropanes, such as cinnamaldehyde, bind with proteins through their carbonyl

	7799	group, preventing the action of important cell enzymes such as amino decarboxylases. Cinnamaldehyde strongly inhibits *C. perfringens* (Gram (+)) and *Bacteroides fragilis* (Gram (−)), but exhibits weak or no inhibitory activity against *Bifidobacterium longum* or *Lactobacillus acidophilus* (Gram (+)). A mixture composed of carvacrol, capsicum oleoresin, and cinnamaldehyde showed antioxidative properties under dietary-induced oxidative stress conditions. The mixture effectively protected the pigs' blood lymphocytes from oxidative DNA damage. Dietary XT (5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin) reduced intraepithelial lymphocyte numbers in jejunum and the percentages of blood cytotoxic cells and B lymphocytes in LN; however, XT increased blood monocytes and the density of lamina propria lymphocytes in the colon.
Other Chinese herbs	**4** Refs: 4563 2500 2630 2632	The Chinese medicinal herbs (CMH) supplementation groups had a higher villus height, increased *Lactobacillus* counts in digesta of ileum, and decreased coliform counts in the colon. The immune activities of polymorphonuclear leucocytes (PMNs), including the respiratory burst and *Salmonella*-killing ability, were significantly enhanced in CMH supplementation groups. CMH supplementation could improve the feed consumption and reduce diarrhoea frequency in weanling pigs. Dietary supplementation with CHM, at the 1% dose, reduced the counts of IEL/100 enterocytes in the duodenum, jejunum, and ileum and increased the number of goblet cells in the duodenum, jejunum, and ileum, decreasing the number of mast cells in the mucosa and submucosa in the duodenum, jejunum, and ileum, respectively. This yielded an increased number of cells per villus positive for serum IgA (sIgA) in the duodenum, jejunum, and ileum, decreased mucosal concentrations of TNF-α in the duodenum, jejunum, and ileum, increased mucosal concentrations of IL-2 in the duodenum and jejunum, and increased mucosal concentrations of IL-4 in the duodenum and jejunum. CHMD as a dietary additive could enhance antioxidant status, serum biochemical parameters, and digestive enzymatic activities in weaned piglets.
Thymus vulgaris derivatives	**3** Refs: 6258 6269 7484	Essential oils (thymol + cinnamaldehyde) reduced the occurrence of diarrhoea and decreased *E. coli* counts in faeces. Feeding EO increased lymphocyte transformation and leucocyte phagocytosis rates as well as the levels of IgA, IgM, C3 and C4 in the blood. The addition of EO improved daily weight gain and feed conversion ratio (FCR) of weaned pigs. Monoterpene phenols, such as thymol and carvacrol, interacted with the cell membrane through H-bonding, rendering the membranes and mitochondria more permeable, disintegrating the outer cell membrane, and particularly inhibiting the growth of Gram (−) bacteria.
Astragalus derivatives	**3** Refs: 2631 2630 2632	In porcine populations, the application of this product as a single substance has not been assessed. The mode of action is the same as that observed in other Chinese herbs (mixture of substances).
Capsicum derivatives	**3** Refs: 6426 3271 7799	Feeding PE (capsicum oleoresin, garlic botanical, or turmeric oleoresin) reduced serum TNF-α and haptoglobin decreased white blood cells and lymphocytes and mitigated villus atrophy after *E. coli* challenge. Pigs whose diets were supplemented with PE often showed protective blood lymphocytes against oxidative DNA damage. Dietary XT (5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin) reduced intraepithelial lymphocyte numbers in jejunum and the percentages of blood cytotoxic cells and B lymphocytes in LN; however, XT increased blood monocytes as well as the density of lamina propria lymphocytes in the colon.
Curcuma longa derivatives	**2** Refs: 4709 6426	Dietary curcumin had no influence on any measured aspect of pig performance or immune status. However, it decreased WBC, neutrophils, lymphocytes, and TNF-α, which indicate that the curcumin may be beneficial for pig growth performance and that it attenuates the systemic inflammation caused by *E. coli* infection. It also reduced the frequency of diarrhoea as well as the average diarrhoea score.
Achyranthes derivatives	**2** Refs: 1761	Dietary supplementation with *Achyranthes bidentata* polysaccharides (ABP) increased ADG and feed efficiency while enhancing humoral immunity in weaned piglets and decreasing diarrhoea frequency in weanling piglets. Dietary supplementing ABP increased plasma concentrations of antibodies and mRNA abundance of IL-1b in the

	1760	liver, the contents of IL-2 and INF-γ, jejunal mucosa GALT, and complement levels. These findings indicate that ABP is effective in improving the growth performance and defending capacity.
Echinacea	**2** Refs: 6605 947	Feed conversion can be improved with the addition of *Echinacea* to diets. Repeated short-time application of *Echinacea* has immune stimulating effects in fattening pigs. It also increased phagocytosis rate and the number of lymphocytes.
Glycyrrhiza	**2** Refs: 2630 5247	The dietary addition of liquorice has no effect on swine growth. The experimental diets containing liquorice activated the intestinal immune system, including the Peyer's patch, as the primary step resulting in higher IgA production in the salivary tissue. Dietary addition of liquorice induced an antinflammation effect in the swine, brought about by a second injection of sheep red blood cells (SRBC) into the dermal tissue.
Sugar cane	**2** Refs: 6443 6444	Sugar cane extract (SCE)-treated pigs showed increased growth. Moreover, SCE has a broad biological effect in raising innate immunity to infections (enhancement of cytotoxic of natural killer (NK) cells and phagocytosis by neutrophils and monocytes), lymphocyte activation, and IFN-γ production that may contribute to the restriction of PrV infection.
Bran derivatives	**2** Refs: 4320 4307	Trends for increased *Bifidobacteria* and improved feed efficiency indicate that stabilised rice bran (SRB) has prebiotic properties. WB (wheat bran) has the ability to interfere in the pathogenic process of the ETEC K88, particularly reducing the adhesion of the pathogen to the epithelium. Pre-treatment of *E. coli* with WB reduced IL-1, IL-8 and TNF-α expression. This reduction could be due to a lower expression of TLR4, inducing decreased stimulation from bacteria. This could be mediated by the previously-shown reduction in the attachment of bacteria. However, these changes could also be due to the indirect immunomodulatory role of WB on the enterocytes.
Pinus derivatives	**2** Refs: 12909 11251	Phytoncide can elevate feed efficiency, increase nutrient digestibility, enhance immune function (serum IgG was greater), and improve the intestinal microbial population (faecal *Lactobacillus* was increased).
Tannin	**2** Refs: 10967 7484	Tannin did not improve the growth performance but also had no deleterious effects on selected blood parameters.
Allium derivatives	**1** Refs: 6426	In animals infected with *E.coli*, *Allium* derivatives reduced average diarrhoea score from d 0 to 2 and d 6 to 11. *Allium* derivatives decreased TNF-α and haptoglobin on d 5, white blood cell (WBC) counts and neutrophils on d 11, and ileal macrophages and neutrophils on d 5. Pigs fed *Allium* also had higher villus height. Therefore, *Allium* may alleviate the overstimulation of the systemic immunity and early immune response of pigs infected with *E. coli*.
Origanum derivatives	**1** Refs: 5748	*Origanum* is supplemented in porcine diets as mixtures with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Beta vulgaris	**1** Refs: 8519	CHO (form of unmolassed sugarbeet pulp) diet induced an up-regulation of IL-6 mRNA content in the colon of piglets 4 d post weaning. An increase in IL-1β mRNA content was also observed on d 4 post weaning.
Citrus by product	**1** Refs: 11251	Limonene is supplemented in porcine diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Syzygium aromaticum	**1** Refs: 11251	Eugenol is supplemented in porcine diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Artemisia	**1**	Additive wormwood (A. Montana Pampan) to the diet stimulated some immune

derivatives	Refs: 1902	responses, including IgM and IgG and IFN-γ concentration of serum and decreased leukocytes, blood urea nitrogen (BUN) and cortisol concentration in the blood of fattening pigs. These results indicate that additive wormwood may benefit pigs, increase their immunity to infection, and may play a role in the reduction of stress.
Pisum sativum	**1** Refs: 8111	Feeding pigs that are weaned early diets containing spray-dried plasma proteins, pea protein isolate, plus egg yolk antibody —or a combination of pea protein isolate and spray-dried plasma protein— after weaning minimises gastrointestinal disorders associated with *E. coli* infection.
Zea mays	**1** Refs: 12177	High-moisture maize fermented with *L. acidophilus* or *P. acidilactici* as a diet supplement was able to modulate pig intestinal microbiota by reducing the diversity and richness of intestinal bacteria.
Solanum tuberosum	**1** Refs: 4987	Feeding of potato protein was effective in linearly reducing the populations of microbes in faeces and the contents of the cecum, colon, and rectum. Feeding potato protein to weanling piglets is an effective means of improving growth performance and reducing pathogenic bacteria.
Linum usitatissimum	**1** Refs: 1916	Probiotic *L. plantarum,* in combination with flax-seed oil rich in n-3 polyunsaturated fatty acids (PUFAs), has anti-inflammatory properties, stimulating Th1-mediated cell immunity and phagocytosis and tending to regulate inflammatory response induced by ETEC.
Trigonella derivatives	**1** Refs: 12866	The piglets fed *Trigonella foenum graecum L.* (fenugreek) had higher *Lactobacillus* and *Clostridium* cluster concentrations and lower *Escherichia*, *Hafnia,* and *Shigella* concentrations in their small intestines. The addition of fenugreek increased the relative concentration of the γδ T-cell population (TCR1+CD8a-) in the blood, with a simultaneous reduction of antigen-presenting cells (MHCII+CD5-).
Agrimonia	**1** Refs: 3801	*Agrimonia procera* extract (APE) reduced the mRNA abundance of tumour necrosis factor (TNF)-α in cells challenged by LPS. After the treatment of PBMC with APE, the relative mRNA concentration of interleukin (IL)-1β declined. Low doses of *Agrimonia procera* may improve the growth performance of piglets and seem to exert anti-inflammatory effects in porcine immune cells challenged by LPS.
Pimpinella anisum	**1** Refs: 5748	Anise oil is supplemented in porcine diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Helianthus tuberosus	**1** Refs: 11708	Feed with the single supplement of Jerusalem artichoke significantly enhances the defence and regeneration processes in the intestine of pigs. Stimulation of β-defensin (2 and 3) production in the intestine through dietary modulation (*Lactobacillus reuteri* and *Pediococcus pentosaceus* with lower dose of Jerusalem artichoke) could promote intestinal health in pigs, thereby reducing the occurrence of infections in pig herds and the subsequent use of antibiotic treatment.
Quillaja saponaria	**1** Refs: 4709	IgG and (C-reactive protein) CRP was greater in saponin-supplemented pigs. Saponin supplementation during the post weaning period seemed to potentiate an immune response in the weaned piglet but had a detrimental effect on the utilisation of feed.
Ceratonia siliqua locust bean	**1** Refs: 4320	The treatment of piglets infected with ETEC K88 with locust bean (LB) reduced the expression of IL-1, IL-8, TNF-α, and TLR-5 but not TLR4. The study demonstrates the ability of LB to interfere in the pathogenic process of the ETEC K88, particularly reducing the adhesion of the pathogen to the epithelium and modifying the innate immune response.

3.4.1.4 Porcine – animal by-product

There are 28 studies concerning animal by-products, and the product most frequently studied was the spray-dried product (SDP) (11 studies).

Table 14: Mode of action of different animal by-products in porcine

Porcine - Animal by-product	Number of articles	Mode of action
Spray-dried product (SDP)	**11** Refs: 3448 12721 11485 12943 809 4555 7799 7800 8332 8111 1717	The effect of SDP on the performance is unclear. SDP improved duodenal villus height and duodenal villus height to crypt depth ratio in piglets. SDP also reduced malondialdehyde content in mucosa. In the mucosa, SDP decreased TNF-α, IL-6, IL-1β, TGF-β, and soluble IL-2 receptor contents. The cytokines in the serum were not affected by SDP. TNF-α and IL-1β mRNA in all tissues and IL-6 mRNA in the adrenal gland, spleen, pituitary gland, and liver were all reduced in pigs fed adiet with SDP compared to those fed a diet without it. Piglets fed porcine SDP (SDPP) diet exhibited higher levels of IgG. Dietary SDP plasma reduced the percentage of blood monocytes and macrophages (SWC3+) in ileal Peyer's patches (PP) and lymph nodes (LN), of B lymphocytes (CD21+) and γδ+ T cells (γδ T cell receptor (TCR)+) in LN, and of intraepithelial lymphocytes as well as the density of lamina propria cells in the colon.
Antibodies	**7** Refs: 6847 1388 5450 1044 1045 1717 8111	Egg-yolk antibodies might protect both neonatal and 21-day-old weaned piglets from diarrhoea induced by a challenge with *E. coli* K88. It is likely that the mechanism by which egg-yolk antibodies protect pigs from ETEC induced diarrhoea is that the antibodies against the fimbriae of ETEC react with the receptor binding component of ETEC (fimbriae), thereby preventing them from attaching and adhering to the mucosa of the intestines of piglets. Pigs fed diets containing egg yolk antibodies had longer villi, less severe diarrhoea, and grew faster. The oral administration of bovine colostrum seems to improve and retain the immunological properties of the structures associated with the gastrointestinal tract in weaning piglets by generating different types of responses protecting the pigs from infectious and allergic diseases. The immunomodulatory effects of bovine colostrum primarily target the GALT, which responds by producing, at different levels, both Th1 pro-inflammatory cytokines (IL-2, IFN-γ and IL-12) and anti-inflammatory Th2 cytokines (IL-4 and IL-10). The CD21+/CD3+ cells populations of the ileal Peyer's patch (PP) were markedly affected, and a decrease in IFN-γ was also observed in the ileal PP consecutive to colostrum administration. Therefore, it is plausible to speculate that bovine colostrum may influence the development of the IgA response by potentiating a Th2 response in the ileal PP.
Lactoferrin (LF)	**5** Refs: 2033 12211 11223 2055 6150	The general increase in cytokine production by mucosal and systemic immune cells isolated from piglets fed dietary bovine lactoferrin (bLF) indicates a priming of the immune system. IL-10 was increased under both unstimulated and LPS-stimulated conditions, which potentially limits inflammation. In LF piglets, the spleen cells, but not MLN, increased with regard to IFN-γ and TNF-α production; MLN, but not spleen cells, tended to increase IL-6 production, demonstrating organ-specific effects of dietary bLF treatment. Strong TNF-α and IL-6 production indicates a robust innate immune response by the cells from piglets fed bLF compared with those fed the control diet. The improved IFN-γ response indicates that dietary bLF may be most important in T-helper-1–type adaptive immune responses and thus is vital to protection from infection. Pigs supplemented with LF had greater villus heights and lower crypt depths at the small intestinal mucosa, which may contribute to improved growth performance. Supplemental LF significantly improved the relative abundance of mRNA for PR-39 and protegrin-1, suggesting that LF can regulate the expression of the 2 cathelicidins in the bone marrow of weanling piglets. LF could regulate transcription of the IL-1β gene and may also regulate transcription of other natural genes containing the LF binding sites. Dietary supplementation with lactoferrin increased recovery from diarrhoea, enhanced serum glutathione peroxidase (GPx), peroxidase (POD) and total antioxidant content

		(T-AOC), liver GPx, POD, superoxide dismutase (SOD) and T-AOC, Fe, total Fe-binding capacity, IgA, IgG and IgM levels ($P<0.05$); meanwhile, it decreased the concentration of *E. coli* in the ileum, caecum, and colon and increased the concentration of lactobacilli and bifidobacteria in the ileum, caecum, and colon, promoting the development of the villus–crypt architecture of the small intestine.
Oils	**2** Refs: 6524 4320 6117	Fish oil may partially improve intestinal barrier function via improving the expression of intestinal tight junction proteins. Fish oil exerts its beneficial effect on the intestine by alleviating the intestinal inflammatory response. The protective effects of fish oil supplementation on intestinal integrity were closely related to the reduction ofthe expression of intestinal proinflammatory cytokines through inhibition ofthe TLR4 signalling pathway.

3.4.1.5 Porcine – other substances

Other substances were investigated in 58 studies. These mainly concern organic acids derivatives and fatty acids (14 studies), minerals (11 studies), and amino acids and their derivatives (19 studies).

Table 15: Mode of action of other substances / agents in porcine

Porcine - Other	Number of articles	Mode of action
Amino acids (AA) and derivatives	**19** Refs: 1406 5373 4671 9014 12593 12995 1389 11515 9015 12199 4543 12162 12721 12951 4972 6499 2983 1795 7390	Feeding AA supplemented diet ameliorated the change in gut morphology and stimulated the expression of ciliary dynein heavy chain 5 in the jejunum, indicating that AA are beneficial to the growth of intestinal mucosa and the movement of villus. The reason for this might be that the specific non- and semi-essential AA are metabolised in enterocytes for proliferation, energy generation, and protection of the small intestinal mucosa. Cysteine significantly reduced the expression of pro-inflammatory cytokines, including TNF-α, IL-6, IL-12p40, IL-1β, resulting in increased expression of the apoptosis initiator caspase-8 and decreasing expression of the pro-survival genes cFLIP and Bcl-xL. IL-8 and MIP-2, implicated in neutrophil and monocyte recruitment, were reduced upon treatment with Cysteine (Cys). Cys supplementation was able to significantly aid the recovery of piglets from colitis. Neutralisation of IL-6 trans-signalling, either with antibodies working against the IL-6 receptor or a gp130-Fc fusion protein, suppressed colitis activity and induced T-cell apoptosis *in vivo*. Growth performance, histological structures, and gut barrier function were improved. In addition to its known anti-oxidant effects, Cys modulated local cytokine gene expression, suppressing pro-inflammatory and chemotactic gene expression and promoting the expression of pro-apoptotic pathways, suggesting that cysteine supplementation may support the recovery of mucosal homeostasis. Treatment of β-glucan alone or plus L-theanine might decrease inflammatory response against Gram (-) bacterial infection via the inhibition of pro-inflammatory cytokine production and the enhancement of anti-inflammatory cytokine production. L-arginine had a positive effect on increasing villus height and the expression of the vascular endothelial growth factor (VEGF) protein. Arginine (Arg) can increase protein synthesis, inhibit protein degradation, and enhance the proliferation of intestinal epithelial cells. Arg supplementation modulated intestinal inflammatory response to LPS by increasing the number of IELs, CD4+ T cells, CD8+ T cells and antibody secreting cells (ASCs) and decreasing Peyer's patch cell apoptosis and mast cell number in the intestinal mucosa of weaned pigs. In F4R+ pigs, the higher standardised ileal digestibility (SID) Tryptophan (Trp): Lysine (Lys) ratio in the diet prevented an increase in the expression of genes involved in the

		innate immune response (regenerating islet-derived 3 gamma (REG3G), Surfactant protein D (SFTPD) and lipopolysaccharide binding protein (LBP)) in the jejunal tissue. Several genes involved in the intestinal barrier appear to be changed by dietary Trp in healthy pigs that are positive for the presence of the intestinal receptors for ETEC. The mechanism by which tryptophan reduces the stimulatory effect of several bacteria-associated molecular patterns in the genes involved in the response of the intestinal barrier in ETEC-susceptible pigs has yet to be established. Dietary SIDT (SID Threonine) increased the relative thymus body weight and duodenal villus width. Serum specific IgG concentrations increased in response to the increased intake of true ileal digestible threonine. IL-1 concentration significantly decreased, and the IL-6 concentration significantly increased in the jejunum of piglets. Supplementation with SIDT improved the intestinal mucosal defensive function after *E. coli* challenge. The protective function of threonine for the rapid production of gut mucosa SIgA under *E. coli* challenge may be mediated through IL-6 regulation. The increase in dietary SIDT level indicated beneficial effects in allowing duodenum villi to widen and jejunal villus height to crypt depth ratio to decrease. In addition, the increased dietary SIDT level was beneficial in alleviating the small mucosa damage caused by *E. coli* challenge. The supplementation of glutamine and glutamine plus glutamate improved feed conversion and reduced villi atrophy in post-weaning pigs. Glutamine was able to alleviate the stressful condition and inflammation and to improve immunity and growth performance in the early starter stage. In response to dietary glutamine supplementation, expression of eight genes was downregulated in the small intestine. These genes are related to cellular signalling transduction [casein kinase 1 epsilon and MAPK-6], the cell cycle [Rho-related GTP-binding protein], apoptosis [KLF-10], immune activation (La antoantigen homolog and ICAM-1), protein modification [peptidylprolyl isomerase G], and gene expression (pre-mRNA cleavage complex II). Enhanced expression of antigens and activation of leukocytes in the intestinal epithelium due to activation of the MAPK-6 signalling may contribute to intestinal dysfunction and diarrhoea in weanling pigs. Another beneficial effect of glutamine supplementation is increased expression of six genes related to transcription regulation [AF-9 protein], lipid metabolism (endozepine), iron absorption (heme-binding protein), cytoskeletal structure and function (myosin), defence against pathological microorganisms [IL-13R-a-1 and endozepine], and regulation of nutrient metabolism (signal recognition particle 72K chain). Feeding glutamine has beneficial effects in alleviating growth depression of *E. coli* K88+-challenged pigs, primarily through maintenance of intestinal morphology and function, and/or possibly through modulation of the somatotrophic axis. Supplementing the diet with Glycine (Gly)-Glutamine (Gln) improved the growth and intestinal integrity of weanling piglets. Gln supplementation during the weaning period is useful in reducing the early stages in weaning-related gastrointestinal infections by suppressing the inflammatory and regulatory cytokine response in the gut and decreasing damage to tight junction proteins and intestinal electrolyte movement. The adverse effects of LPS on intestinal integrity could be mediated by the decline in average daily feed intake ADFI, but could also be directly affected as a result of a systemic inflammatory response (IL-1β secretion), whereas Gly-Gln may have the ability to limit this proinflammatory response. Reduced performance was observed in glycinin- or b-conglycinin sensitised piglets. The Increased Proliferative Index, Apoptotic Index, and relative metabolic rate (REMR) were only observed in duodenum of piglets fed with glycinin or b-conglycinin. Glutamate supplementation numerically improved duodenal villus height and IgG levels.
Organic acids derivatives / Fatty acids	**14** Refs: 8312 809 3510 6286 12123 6082 3045 13177 519	Organic acids improved growth performance primarily via reducing pathogenic bacteria and increasing beneficial bacterial population in the gut and faeces. Diets supplemented with organic acids fail to affect the growth performance of weaned pigs. Diets supplemented with organic acids enhanced PBMC and MLN proliferation by Concanavalin (Con) A. Poke weed mitogen (PWM) stimulation was more evidenced than the organic acids effect on spenlocyte proliferation, Ig or antiSEP titer response. The mechanisms for the effects of diet supplemented with organic acids on enhancing T- and B- cell proliferations were dependent on energy supply, and other pathways still need to be understood. Piglets weaned from conjugated linoleic acid (CLA)-supplemented sows had superior intestinal health and immune status markers, as indicated by reduced intestinal mucosal inflammation and elevated serum IgG and IgA. CLA delayed the onset of experimental

	521 1901 734 3059 6414	IBD and attenuated growth suppression in pigs by activating the colonic peroxisome proliferator-activated receptor (PPAR)-g, whereas n-3 PUFA accelerated remission by activating PPAR d. Furthermore, mixtures of CLA and n-3 PUFA accelerated recovery by activating PPAR d, i.e. induced uncoupling protein (UCP)-3 and upregulating the expression of KGF in the colon. CLA-supplementation increased the numbers of γδ+-T cells in the colonic mucosa in pigs with bacterial-induced colitis. CLA favoured a CD8+ T cell phenotype with an increased predominance of naive cells (CD8+CD29low and CD8+CD45RC+). This naive CD8 Tcell phenotype in the peripheral blood of uninfected pigs fed CLA originated from an enhanced thymic output of CD8 T-cells. After the PCV2 challenge, this was overshadowed by the expansion of antigen-specific CD8 T-cells and increased CD25 expression. In T-cell immunoregulation, dietary CLA differentially modulated PPAR-α and PPAR-γ expression in PCV2-infected pigs. Greater expression of PPAR-α in the lymph nodes of pigs fed with the control diets was associated with attenuated CD8+ T-cell responses. Conversely, the enhanced PPAR-γ expression in the lymph nodes of pigs fed CLA was linked to increased CD8 responses. Microencapsulated organic acids (MOA) group (20% citric acid, 20% fumaric acid, 10% malic acid, and 10% phosphoric acid) IGF-I concentrations declined from preinfection concentrations on d 2, increased on d 4, and declined again until d 13. The serum concentrations of the cytokines IL-6 and IL-1β were not generally affected by SalT challenge. Dietary Sodium Butyrate (SB) could regulate and enhance the immune function of piglets by increasing the serum IgG concentration and IgA+ cell count in jejunum. SB may reduce the adverse effects of weaning stress and may play an important role in maintaining the integrity of intestinal mucosa. Acidification of the diet with citric acid positively affected the feed conversion ratio and reduced pathogenic bacterial load. Dietary supplementation of citric acid increased beneficial bacterial counts and serum IgG concentration compared to the control group. Giving alkylglycerol fatty acids to sows in late gestation and when lactating can improve passive immunity transfer to piglets. Feeding flax (long-chain n-3 fatty acids) to sows can have beneficial effects on litter performance; specific effects can differ depending on whether it is fed as seed, meal, or oil. Feeding flax in any form to sows in late pregnancy and those that are lactating enhanced the immune response of piglets, which may explain the increased survival rate of these piglets. The addition of citric acids to the basal diets during late gestation and lactation did not affect sow and litter performance but could improve the utilisation of crude protein (CP), calcium (Ca), and phosphorus (P) as well as the immunoglobulin levels of plasma, colostrum, and milk.
Minerals	**11** Refs: 1406 7196 3428 10037 4866 4930 1235 1570 3220 6243 1795	Zinc (Zn) inhibits the induction of NF-κB in response to pathogens, possibly through heat shock proteins. Reduced intestinal expression of NF-κB target genes and subsequent inflammation may result in reduced tissue damage and may impact infection. Maintenance of normal gut function through reduced inflammation could result in an improvement in piglet performance, as seen with dietary zinc oxide (ZnO) treatment. Supplementation of feed with Zn immediately after weaning could positively affect the immune responses of piglets infected with *Salmonella Typhimurium*, but only for a period. After two weeks, all positive effects disappeared, and negative effects such as higher shedding of salmonellae, lower T-cell frequencies, and worse performance, began to occur. Zinc supplementation had no effect either on the number of excreted *E. coli* and enterococci per gram of faeces or on the functions of circulating neutrophils, but it increased the growth of the piglets during the first two weeks postweaning. Zinc oxide reduced bacterial translocation to the MLN, and this may have resulted from immune system modulation and enhanced intestinal IgA concentration. Bioplex Zn tended to increase the weight of the ileum containing Peyer's patches. ZnMet increased serum and liver Zn concentrations, the number of goblet cells, length of jejunal villus epithelium, and tended to enhance jejunum mucosa thickness. Interactive effects for higher jejunal villi height and villi:crypt ratio and increased ileal goblet cell counts were apparent for pigs from ZnAA-supplemented sows that also received nutriment-intubation of ZnMet. The selenium (Se) enriched probiotics (SP) improved growth performance of piglets. Both selenium selenite (SS) and SP increased blood glutathione peroxidase activity and tissue

		thioredoxin reductase 1 mRNA expression, with SP being higher than SS. All P, SS, and SP supplementation increased the superoxide dismutase activity, glutathione content, TCR-induced T lymphocyte proliferation, and IL-2 concentration and decreased malondialdehyde content. The beneficial effects may be explained by two possible reasons: (i) there is an additivity or a synergistic effect between probiotics and Se, and (ii) the more robust antioxidant effects of the SP product may be due to the organic Se it contained relative to the inorganic Se contained in the SS. The potential effect of metallic silver as a dietary additive on intake and growth of weaned piglets could be mediated through its antimicrobial properties, either against certain bacterial groups or reducing the microbial load of the small intestine. Animals feeding diets with Sericite showed increased blood IgG concentration, lymphocyte, and monocyte percentage and decreased faecal *E. coli* population counts. Sericite also increased faecal *Lactobacillus* counts.
Algae	**6** Refs: 2600 11251 12118 2599 5247 6186	*A. nodosum algae* showed potential, with regard to weaned piglet nutrition, as a feed material for improving gut flora; this is an important index of the gastro-intestinal health status. Inflammation-related cytokines, such as IL-1β, IL-6 and TNF-α, can be said to have a negative effect on the growth and health maintenance of swine. The dietary addition of liquorice induced an antiinflammation effect in swine. Seaweed treatment induced high expression of IL-2 and IL-4, which enhances humoral and cellular immune functions, possibly resulting in increased SRBC-specific IgG and increased DHRs. A high concentration of IgA in the saliva of both the seaweed- and liquorice-treated groups was observed. Mucosal IgA played a role in protecting the mucosal epithelial cells from infection by bacteria and neutralising bacterial toxins. Activation of the Peyer's patch induces the expression of IL-5 and IL-6, which in turn enhances the differentiation of B-cells into plasma cells. The activated plasma cells produce IgA in the intestinal mucosa, resulting in enhanced effects on the intestinal immune system. As a consequence of the activation of the intestinal immune system, increased IgA production in other types of mucosal tissues (oral, nasal, and bronchial mucosa) would be induced by the circulation of the activated plasma cells throughout the body. Maternal dietary treatment (seaweed extracts (SWE) + fish oil (FO)) improved small-intestinal morphology and reduced caecal *E. coli* populations in pigs. Interestingly, maternal SWE and FO supplementation up-regulated intestinal pro-inflammatory cytokine expression; however, no deleterious effects were observed on performance. Fucoidan (FUC) diet singularly had an increased villus height and villus height to crypt depth ratio compared with pigs offered the basal diet. Pigs offered the laminarin (LAM)-supplemented diets had lower IL-6, IL-17, and IL-1β mRNA expression in the colon compared with pigs offered diets without LAM supplementation.
Nucleotides	**4** Refs: 6082 10075 1797 7390	Diets supplemented with organic acids and nucleotides failed to affect the growth performance of weaned pigs. Dietary supplementation with organic acids and nucleotides had a synergetic effect on the Peyer's patches and mesenteric lymph node lymphocyte proliferation. The diets supplemented with nucleotides increased the bile and plasma IgA levels and modulated the gastrointestinal tract in weaned pigs. Diets supplemented with 0.5% nucleotide base also increased PBMC proliferation. Supplementing the diet of weaning piglets with pure nucleotides resulted in an increase in plasma IgA concentrations without altering gut morphology, bacterial numbers, and growth performance. Nucleotide supplementation numerically improved duodenal villus height. Treatments with CpG oligodeoxynucleotide (ODN) reduced bacterial load in the phases at days 3–5 post challenge. The chemokine (CXC) (CXCL10 and CXCL11) and CC chemokine (CCL4 and CCL5) mRNA expressions were elevated in the intestinal tissues from animals treated either intranasally or orally with CpG ODN, compared to untreated controls. This significantly enhanced mRNA expressions for cathelicidins (PR-39 and protegrin-1) and did so moderately for β-defensin (pBD1 and pBD2), as observed in CpG-treatments. Additionally, significant production of cytokines (IL-12, IFN-γ, and MCP-1) and F4-specific antibodies (IgG/IgA) was detected in intestinal washing, following intranasal and oral CpG-treatment.
Vitamins	**3**	Vitamin E supplemented to pigs can effectively protect their blood lymphocytes against oxidative DNA damage, thus suggesting potentially beneficial effects on the immune

	Refs: 3271 6009 7390	system under dietary-induced oxidative stress. Dietary fat source, rather than vitamin E supplementation after weaning, influenced the measured responses of the pigs, i.e. humoral immune response, plasma concentration of triglyceride and cholesterol, and fatty acid composition of mucosal samples.
Peptides	**3** Refs: 12749 12532 12750	The results of the studies indicate that piglets fed a diet containing the antimicrobial peptide cecropin AD showed improved performance and reduced incidence of diarrhoea. The beneficial effects of cecropin AD post *E. coli* challenge in weaned piglets appears to be mediated through changes in immune status. Antimicrobial peptide (AMP)-A3 has the potential to suppress harmful intestinal microflora in weanling pigs. AMP-A3 had no effects on serum immunoglobulin concentrations. The results indicate that improved growth performance in the studies is not due to enhanced immune function but rather might be due to improved gut barrier function via exclusion of pathogenic microorganisms (*Clostridium* and coliforms) and improved intestinal morphology. AMP-A3 showed beneficial effects on growth performance, coefficients of total tract apparent digestibility (CTTAD) of dry matter (DM) and crude protein(CP), intestinal morphology, and intestinal and faecal microflora.
Lactulose	**2** Refs: 3937 8519	Lactulose supplementation at 10 g/kg of the diet increased growth performance and modified fermentation in the colon with reductions of the blood urea nitrogen.

3.4.2. Poultry

The most common substances and agents studied with regard totheir effects on the immunity of poultry were probiotics and prebiotics (Table 6).

3.4.2.1 Poultry – probiotic

The systematic review reported 373 studies of probiotics in poultry. Most of these were regarded as *Lactobacillus spp.* (113 studies), *Bacillus spp.* (59 studies), *Enterococcus spp.* (53 studies), *Bifidobacterium spp.* (47 studies), *Saccharomyces spp.* (29 studies), *Streptococcus spp.* (26 studies), *Aspergillus spp.* (16 studies), *Pediococcus spp.* (10 studies), *Clostridium spp*. (9 studies), and other minor groups (Table 16).

In general terms, the supplementation of poultry diets with *Lactobacillus spp.* (113 studies) improves the performance of broilers and decreases the numbers of coliforms in the cecum 10 and 20 days after feeding. *Lactobacillus* also significantly increased the total VFA in the ileum and cecum and reduces the caecal pH values. Increments in the villus height (VH) and crypt depth (CD) of chickens fed *Lactobacillus spp*. supplemented diets could improve the absorptive surface area in the GI tract. The *Lactobacillus spp*. birds showed increased goblet cell (GC) numbers, total GC area, GC mean size and mucosal thickness. *Lactobacillus spp*. has an immunoregulatory effect of dietary probiotics of the local immune system in broiler

chickens. Birds with the *Lactobacillus* diet showed an increase in intra-epithelial lymphocytes (IEL) expressing the surface markers CD3+, CD4+, CD8+, and αβTCR. *Lactobacillus spp*. induces IL-1, IL-12p40, IL-10, IL-18, TGF-4, and IFN-γ in gut-associated lymphoid tissues of chicken. The immunomodulatory action (inflammatory / anti-inflammatory response) of *Lactobacillus* is dependent on the characteristics and quantity of the strain involved. *Lactobacillus spp*. was also efficient in stimulating the production of total intestinal and serum IgA and IgG.

The supplementation of poultry diets with *Bacillus spp*. (59 studies) improves performance and increases the relative weight of immune organs. *Bacillus spp.* also improved gut health and integrity by increasing the villus height as well as the villus height to crypt depth ratio. Dietary supplementation with *Bacillus spp*. can potentially alter gut microflora by selectively stimulating the growth of beneficial bacteria while suppressing the growth of pathogenic bacteria. Dietary *Bacillus spp.* lowered IFN-γ, IL- 1β, and CXCLi2 cytokine mRNA expression in chickens. *Bacillus spp.* downregulated IL-2, IL-4 and IL-6, favouring an anti-inflammatory response in the gut. This indicates that *Bacillus* had both local and systemic immunity effects. *Bacillus spp*. was also efficient in stimulating the production of total intestinal and serum IgA and IgG.

The supplementation of poultry diets with *Enterococcus spp*. (53 studies) improves growth performance and reduces the counts of pathogenic bacteria in the gut; it also reduces the spread of pathogens. *E. faecium* also plays a beneficial role in jejunal morphology and may reduce oxidative damage. The *Enterococcus* strain can increase phagocytosis as well as strengthening non-specific immunity. *Enterococcus spp*. was primarily used as a component of a multristrain mixture of probiotics and other substances.

Bifidobacterium spp. (47 studies) was used primarily as a component of a mixture of probiotics and other substances. Therefore, the modes of action described in the literature usually include mechanisms induced by this mixture and, as a consequence, the description of the mode of action could not be identified as a single substance.

S. cerevisiae (29 studies) improves performance, immune function, and intestinal mucosal morphology of broilers. Yeast supplement in the diet also increases the numbers of lactobacilli and decreases the numbers of total aerobic bacteria, coliforms,

Enterobacteriaceae, and *Enterococci*. Supplementation of *S. cerevisiae* fermentation product increased CD3+, CD4+, and CD8+ T-lymphocyte counts and the ratio CD4+:CD8+ in the blood and spleen as well as ileum intraepithelial lymphocyte count, caecal tonsil secretory IgA counts, serum lysozyme content, and albumin:globulin ratio. Gene expression levels of major histocompatibility complex (MHC)-II, CD40, CD80 and CD86 up-regulated in stimulated groups with *S. boulardii*. Furthermore, toll-like receptors TLR1, TLR2, TLR4, and chicken specific TLR15 expressions were improved. Downstream associated factors of myeloid differentiation (MyD)88, TNF receptor-associated factor 6 (TRAF6), TGF-β activated kinase 1 (TAK1), and NF-κB mRNA levels increased in all treatment groups as compared to the control groups; IL-1b, IL-17, IL-4, TGF-β, and IL-10 production levels were higher. Lower concentrationof INF-γ and IL-8 were observed in *S. boulardii*.

Table 16: Mode of action of different probiotics in poultry

Poultry-Probiotic	Number of articles	Mode of action
Lactobacillus spp.	**113** Refs: 5592 5239 9063 11818 7422 10723 10755 10724 8360 10722 8359 3221 845 10721 406 10388 7457 7631 45 12780 4042 12781 9047 6402 3582 9465 3585 11555 4368 1066 4370 5491 849 408 7460 7126 4044 12080 10468 7817 4984 7818 1481 11944 4366 13040 2212 2226 356 4369 9155 1727 1711 5403 2249 3559 2251 3060 10469 2316 1222 11477	Supplementing the *Lactobacillus* cultures, singly or in a mixture, in the diet of broilers significantly increased the body weight and feed:gain ratio of broilers significantly decreased the number of coliforms in the cecum 10 and 20 days after feeding. *Lactobacillus* also significantly increased the total VFA in the ileum and cecum and lowered the caecal pH values. The DFM birds showed increased goblet cell (GC) numbers, total GC area, GC mean size, mucosal thickness, and an increased number of segmented filamentous bacteria compared with controls. Also, the level of MUC2 mRNA in both the jejunum and ileum were increased. Increments in VH and villus height: crypt depth (VH:CD) ratio was directly correlated with enhanced epithelial cell turnover. This could mean that the increments of VH observed with DFM treatment were associated with increased enterocyte turnover rates. Increments in VH and CD of the DFM-treated poults could improve the absorptive surface area in the GI tract of these poults, potentially leading to improved performance. Supplementation of *Lactobacillus* recruits a gut environment favourable for the colonisation and growth of certain groups of lactobacilli, inducing a beneficial microbiota. *Lactobacillus spp*. has an immunoregulatory effect of dietary probiotic on the local immune system in broiler chickens. Birds with *Lactobacillus* diet showed an increase in IEL expressing the surface markers CD3, CD4, CD8, and αβ-TCR. The duodenum, followed by the jejunum, were the segments in which the immune response by T, CD3+, CD4+, and CD8+ cells was stimulated with the strongest intensity. The strains of *Lactobacillus spp*. were efficient in stimulating the production of total intestinal and serum IgA and IgG. The expression of STAT2, STAT4, IL-18, MyD88, IFN-α, and IFN-γ genes were up-regulated in caecal tonsil cells after treatment with *Lactobacillus* DNA, suggesting development of a Th1 phenotype. *L. acidophilus*, *L. reuteri* and *L. salivarius* induced significantly more IL-1 expression in spleen cells than in caecal tonsil cells, indicating a more inflammatory response in the spleen than in caecal tonsils. In

	4576 4502 8812 7461 3747 11152 12934 7650 4043 12666 13204 1826 8754 1827 10667 46 1161 3862 9189 12442 1220 12440 1221 2264 6234 3561 6267 5346 249 7737 5252 8175 4367 7604 839 7893 11151 235 4985 603 992 5000 1483 10783 468 8813 10066 12653 10959 10615 11849	caecal tonsil cells, substantial differences were found among strains in the capacity to induce IL-12p40, IL-10, IL-18, transforming growth factor 4 (TGF-4), and IFN-γ. *L. acidophilus* is more effective at inducing T-helper-1 cytokines while *L. salivarius* induces a more anti-inflammatory response. Effects of multistrain probiotics are associated with changes in cytokines expression, such as IL-1β, IL-6, IFN-γ, and IL-10, in the gut-associated lymphoid tissues of the chickens; this correlates with protection against both the viscera invasion and gut colonisation by *Salmonella typhimurium.* The immunomodulatory action of *Lactobacillus* is dependent on the characteristics and quantity of the strain involved.
Bacillus spp.	**59** Refs: 4516 4560 12087 135 2396 4938 6172 13178 154 11958 2382 6347 5549 7220 10755 6122 2502 5548 7360 12935 8224 12511 10286 10287 4906 12932 1742 1770 8360 8359	*B. subtilis* to broilers could improve growth performance as well as increasing the relative weight of the thymus. *Bacillus* also improved the gut health and gut integrity by increasing the villus height and villus height to crypt depth ratio. *B. subtilis* spores in broiler chicken feed may exert beneficial effects by one or more of these mechanisms: (1) oxygen consumption — creating a more favourable environment for beneficial anaerobic species; (2) competitive exclusion in limiting the colonisation of some pathogenic bacteria, especially *E. coli, Salmonella enterica, Clostridium perfringens*; (3) enzyme production; (4) enhanced immune response; (5) activation of intestinal function. The increase in diffuse lymphohistiocytic infiltration in the mucosa, and the increased number of solitary lymphoid follicles occurring in the connective tissue layer of the mucosa, paralleled increasing *B. subtilis* concentrations of the feed. The increase in solitary lymphoid follicles, indicating gradual lymphoblast cell production, is suggestive of increased immunological activity of the mucosa. Dietary supplement with probiotics can potentially alter gut microflora by selectively stimulating the growth of beneficial bacteria while suppressing the growth of pathogenic bacteria. *Bacillus* induced an increase in the concentration of serum IgG and IgA. The effects may be related to activation and maturation of epithelial immune cells, stimulation of heterophil bactericidal mechanisms, and increases in the activity and number of T-cells and B-cells, leading to increased synthesis of immunoglobulins. Cytokine mRNA expression was significantly altered by *Bacillus spp.* Of interest, dietary DFMs lowered interferon-γ, IL-1β, and CXCLi2 cytokine mRNA expression in chicken. IL-2 and IL-4 are produced by naïve and T-helper 2 cells, respectively, in response to antigenic stimulation; on activation by antigen recognition and stimulation naïve T-cells produce IL-2, which bind to its receptor, initiating proliferation of T-cells that recognise the antigen. Chicken IL-6 is secreted by T-cells and macrophages and acts as both a pro-inflammatory in

	10499 6124 6127 6128 45 6525 3582 10468 10469 12934 6267 9465 5403 3060 8827 8828 5346 12763 3345 4518 10376 9505 2497 6169 13082 12479 8830 10434 1771	association of the production of acute phase proteins and anti-inflammatory cytokine. *Bacillus spp*. downregulated IL-2, IL-4 and IL-6 favouring an anti-inflammatory response in the gut. *Bacillus spp*. downregulated caecal tonsil IL-2, IL-4, and IL-6, and splenic IL-2 and IL-4 expression. This indicates that *Bacillus* had both local and systemic immunity effects as well as increased ileal and caecal tonsil expression of TGF-β4. The cell surface receptors TLR1, 2, 4, and 15 showed significant up-regulation at mRNA levels. In addition, associated factors MyD88, TRAF6, TAK1, and NF-κB were found to respond to all treatment groups. *Bacillus* can trigger the MyD88-dependent signalling pathway and activate the immune system by stimulating TLRs and, subsequently, TRAF6 expression up-regulation.
Enterococcus spp.	**53** Refs: 406 7457 45 9047 402 1477 4315 6557 3582 3585 4094 7460 13204 8754 10667 1161 6234 10723 10724 10722 3221 10388 11555 1727 3559 2316 11477 4502 7461 11152 7650 12666 1826 1827 46 3862 12442 12440 2264 3561 3562 4181 6217 8571 12933 1487 235 603 6819 8813 10587 6818 1408	*E. faecium* enhanced weight gain and feed conversion in broilers, efficiently inhibiting the adhesion of *E. coli* to the intestinal mucus, likely through steric hindrance, altered pH, and increases in villus height in broilers. *E. faecium* plays a beneficial role in the jejunal morphology. The application of *E. faecium* reduced colonisation of caeca and minimised translocation of *Salmonella* into the liver and spleen, which plays an important role in a reduction of the pathogen spread. *E. Faecium* is thought to influence the dynamics of intestinal mucin production in birds, which is supported by the beneficial effect of *E. faecium* on chickens infected with *S. Enteritidis.* These chickens have been shown to have a higher number of peripheral blood cells and an increased frequency of lymphocyte subpopulations in the blood at first sampling. *E. faecium* also increased the number of IgM+ cells after the administration of *S. Enteritidis*. The administration of the probiotic strain *E. faecium* AL41 may cause minor oxidative damage, e.g.intracellular accumulation of short-chain fatty acids produced by the bacteria, thereby inducing increased capacity of antioxidant enzymes. The probiotic strain AL41 can increase phagocytosis and strengthen non-specific immunity. The addition of the probiotic strain *E. faecium* to the feed of ISA Brown hens reduced leucocyte counts and haematocrit values in blood plasma while erythrocyte counts were increased. The administration of *E. faecium* strains reduced the levels of spoilage microorganisms, such as *E. coli* and *Pseudomonas*, in the digestive tract of turkeys. The feeding of EK13 strain increased the body weight of turkeys. *Enterococcus spp.* was typically used as a component of a multristrain of probiotics and other substances.

Bifidobacterium spp.	**47** Refs: 8360 8359 5087 845 7457 45 4042 9047 3582 3585 7460 4044 4043 13204 8754 10667 1161 6234 10723 10724 10722 3221 10721 10388 11555 3559 3060 2316 11477 4502 7461 11152 7650 12666 1826 1827 46 3862 12442 12440 2264 3561 8175 235 603 10265 8813	*Bifidobacterium spp.* was typically used usually as a component of a multristrain of probiotics and other substances. The modes of action described in the literature usually include the mechanisms induced by a mixture of probiotics, prebiotics, and other substances. The *Bifidobacteria* population in chickens fed a diet containing GOS and *B. lactis* significantly increased 21-fold in comparison to the control-fed birds. In particular, increasing the dietary concentration of GOS was accompanied by significant increases in bifidobacteria counts. The detectable population of bifidobacteria was also greater in chickens fed the diet containing GOS and bifidobacteria when compared with chickens fed a bifidobacteria-containing ration only. Probiotic administration has been shown to reduce *Salmonella* colonisation in broilers. The increasing IgA and IgG concentrations in plasma and intestinal tissue with broiler age confirmed the maturation of the immune system of broilers with time post-hatch. Dietary administration of a direct-fed microbial was found to have the potential to reduce numbers of *C. perfringens* in the GI tract of turkey poults infected with this bacterium. The probiotic product resulted in a beneficial modulation of the caecal microflora, as evidenced by the significant increases in the concentrations of bacteria belonging to *Bifidobacterium spp.*, *Lactobacillus spp.*, and gram (+) cocci in treatments.
Saccharomyces spp.	**29** Refs: 2382 10468 2212 10469 9189 6234 5252 839 992 468 9465 2226 6134 5403 8827 8828 3576 9055 12874 7559 5346 9390 3444 1742 3443 6184 10783 12653 1771	*Saccharomyces spp.* was typically used as a component of a multristrain of probiotics and other substances. The modes of action described in the literature usually include the mechanisms induced by a mixture of probiotics, prebiotics and other substances. *S. cerevisiae* improves growth performance and affects immune functions, Ca and P digestibility, and intestinal mucosal morphology of broilers. Growth performance varied with the levels of yeast supplemented, and immune function could be modified with dietary yeast supplementation. Supplementation of *S. cerevisiae* fermentation product increased CD3+, CD4+, and CD8+ T-lymphocyte counts and the ratio CD4+:CD8+ in blood and spleen as well as ileum intraepithelial lymphocyte count, caecal tonsil secretory IgA counts, serum lysozyme content, and albumin:globulin ratio. Gene expression levels of MHC-II, CD40, CD80 and CD86 up-regulated in stimulated groups with S. boulardii. Furthermore, toll-like receptors TLR1, TLR2, TLR4, and chicken specific TLR15 expressions were improved, and downstream associated factors MyD88, TRAF6, TAB1, and NF-κB mRNA levels increased in all treatment groups as compared to control groups. IL-1β, IL-17, IL-4, TGF-β, and IL-10 production levels were found to be higher, and lower concentration of INF-γ and IL-8 were observed in *S. boulardii*. Addition of kefir as a *Lactobacillus*-yeast supplement in the diets of goslings significantly increased the number of *Lactobacilli* considered to provide balanced microflora in the intestine; this significantly decreased the number of total aerobic bacteria, coliforms, *Enterobacteriaceae*, and *Enterococci*. Feeding geese with *S. cereviae* + *B. subtilis* can improve growth and feed intake and can modulate the intestine ecology, increasing *Lactobacillus* and decreasing *E. coli*.
Streptococcus	**26**	*Streptococcus spp.* was typically used as a component of a

spp.	Refs: 7422 4560 8360 8359 4042 4044 4043 9189 6234 5252 10723 10724 10722 3221 10721 10388 2226 3060 2316 11477 4502 12763 8175 235 603 10783	multristrain of probiotics and other substances. The modes of action described in the literature usually include the mechanisms induced by a mixture of probiotics, prebiotics, and other substances. Chickens given probiotics (*S. faecium* + *L. acidophilus*) showed a reduction in the frequency of *C. jejuni* shedding in colonised chicks and a reduction in jejunal colonisation in colonised chicks. Greater villus height was obtained in the duodenum, jejunum, and ileum with the use of probiotics and prebiotics, and greater crypt depth was obtained with the use of probiotics. Administration of probiotic mixtures containing *Lactobacilli*, *Bifidobacteria*, and *Enterococci* to broiler chicks resulted in a larger quantity of mucosal-adherent bacteria in the small intestine, accompanied by a greater number of ileal goblet cells and a larger mucous layer. When treated with these probiotics, immunised birds mounted a significantly greater antibody response (predominantly of the IgM isotype). Probiotics may enhance the specific antibody response to a thymus-dependent cellular antigen. It is possible that these effects are mediated by cytokines secreted by immune system cells stimulated with probiotic bacteria. In this regard, Th-2 cytokines, such as IL-4 and IL-10, play an important role. Probiotics increased the population of IL-6 cells in the cecum and colon mucosal tissues of chicks. Probiotics (*Streptococcus faecalis, Clostridium buthricum, and Bacillus mesentericus*) cause an influx of the CD8+ T-cells into the intestinal mucosa, which may enhance intestinal immunity by CD8+ T-cells in young chicks. IL-12 and IFN-γ expression is associated with probiotic-mediated reduction in intestinal colonisation with *Salmonella typhimurium*. *Lactobacillus* + *Streptococcus* + *Saccharomyces* stimulate the lysozyme level, complement activity, phagocyte index, and phagocyte count in turkeys.
Aspergillus spp.	**16** Refs: 11160 8360 8359 10468 10469 6234 10723 10724 10722 2316 11477 4502 5346 8175 235 603	*Aspergillus spp*. was typically used as a component of a multristrain of probiotics and other substances. The modes of action described in the literature usually include the mechanisms induced by a mixture of probiotics, prebiotics, and other substances. *Aspergillus oryzae* feeding has an anti-inflammatory action and changes to IFN-γ, IL-1β and TLR-4 mRNA in immune-related cells of the gut in these chicks; this appears to be similar to the effects in those in the antibiotics fed group. In order to obtain better duodenal morphometry, characterised by higher villi, probiotics containing more than one bacterial culture is required (*Bacillus, Lactobacillus, Streptococcus, Bifidobacterium* and *Aspergillus*). Greater VH was obtained in the duodenum, jejunum, and ileum with the use of probiotics and prebiotics and greater CD with the use of probiotics, in relation to the control group. Birds fed the probiotic (*Bacillus, Lactobacillus, Saccharomyces, Aspergillus*) diet showed improved overall weight gain and CP retention, higher *Lactobacillus* and *Bifidobacterium* in the caecum, and reduced *Clostridium* and coliforms in the caecum. The inclusion of these probiotic mixtures into the diet generally improves the oxidative stress and increases the lymphoid organ weights.
Pediococcus spp.	**10** Refs: 7457 849 7460 3747 11555 6134 3559	*Pediococcus spp.* was typically used as a component of a multristrain of probiotics and other substances. The modes of action described in the literature usually include the mechanisms induced by a mixture of probiotics, prebiotics, and other substances. Some strains of *Pediococcus* species produce antimicrobial peptides (bacteriocins) that inhibit closely-related lactic acid bacteria and other Gram (+) spoilage and pathogenic bacteria. These bacteriocins are designated pediocins, and they have been shown to exert high antimicrobial activity against *Listeria* species. Additionally, live *P. acidilactici* bacteria provided some degree of defence against *E.*

	6140 3060 7461	*acervulina* and *E. tenella* infections in broiler chickens, enhancing the humoral immunity against coccidiosis. *Pediococcus* and other probiotics (*Bacillus, Lactococcus, Lactobacillus, Bifidobacterium* and *Streptococcus*) can significantly improve heterophil oxidative burst and degranulation in broilers. The administration of the multispecies probiotic product containing avian-derived *Enterococcus, Pediococcus, Lactobacillus,* and *Bifidobacterium* microorganisms to broiler chickens reduced the caecal pathogen colonisation (such as *C. jejuni* and *Salmonella*) and may change their gut microflora in a way that is beneficial to the health of consumers by reducing the number of potential food-borne pathogens. The probiotic mixtures with *Pediococcus* also modulate intestinal mucin monosaccharide composition, mucus layer thickness, and intestinal morphology in broilers.
Clostridium spp.	**9** Refs: 4516 4560 1770 1476 12626 12895 12934 12763 4518	*C. butyricum* improves growth performance. *C. butyricum* used as a probiotic can modulate nitrogen metabolism, perfect intestinal morphology, and balance the caecal microflora in broiler chickens. *C. butyricum* promoted serum IgA, IgG, and IgM. *C. butyricum* inhibited the growth of caecal pathogenic bacteria, such as *E. coli, Salmonella*, and *C. perfringens*, and promoted the growth of *Lactobacillus* and *Bifidobacterium*. Dietary *C. butyricum* induced higher acetic acid, butyric acid, valeric acid, and total short-chain fatty acids in the caecal digesta of broiler chickens. This, in turn, promoted the growth of beneficial bacteria and lowered the pH of caecal digesta, which inhibited the growth of pathogenic bacteria. A probiotic mixture (*Streptococcus, Clostridium* and *Bacillus*) increased the population of immunoreactive IL-6 cells in the cecum and colon mucosal tissues of chicks. As IL-6 is a multifunctional cytokine for the immune system, the increased IL-6 secretion may affect the immune functions in the intestinal mucosal tissues. Probiotics consisting of *Streptococcus faecalis, Clostridium buthricum,* and *Bacillus mesentericus* caused a significant influx in the CD8+ T-cells. Probiotics have an enhancing effect on intestinal immunity by CD8+ cytotoxic T-cells to protect young chicks from infections.
Candida spp.	**9** Refs: 10723 10724 10722 2316 11477 4502 8175 235 603	*Candida spp.* was typically used as a component of a multristrain of probiotics and other substances. The modes of action described in the literature usually include the mechanisms induced by a mixture of probiotics, prebiotics, and other substances. Dietary supplementations of probiotic mixture (*Lactobacillus plantarum, L. acidophilus, L. bulgaricus, L. rhamnosus, Bifidobacterium bifidum, Streptococcus thermophilus, Enterococcus faecium, Aspergillus oryzae,* and *Candida pintolopesii*) boosted humoral immune response that might be due to lower cortisol concentration or the stabilising of lactic acid-producing bacteria augmenting the production of anti-inflammatory cytokines and immunoglobulins and enhancing the macrophage phagocytic activity in the intestinal lymphoid tissues. Pro-inflammatory cytokines, particularly IL-6, are responsible for the overexpression of C-reactive protein (CRP) in response to various stimuli. An improvement in body weight gain (BWG) and feed efficiency (FE) in the probiotic mixture groups may be attributed to the total effects of probiotic bacterial action, including improved intestinal absorption, resistance to disease, and promotion of host-beneficial bacteria.
Lactococcus spp.	**1** Refs: 3060	*Lactococcus in vitro* was capable of increasing heterophil oxidative burst and degranulation. The data suggest that a rapid change in gut microflora significantly increased heterophil function.
Propionibacterium	**1**	*Propionibacterium* is supplemented in poultry diets as a mixture with

spp.	Refs: 12087	other probiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.2.2 Poultry – prebiotics

The systematic review indicated 130 studies of prebiotics in poultry. Most of these concerned mannan oligosaccharides (MOS; 48 studies), the yeast cell wall (YCW; 22 studies), glucan (19 studies), fructooligosaccharides (FOS; 13 studies), inulin (10 studies), and other minor groups (Table 17).

Adding MOS to poultry diets (48 studies) has indicated improvements in performance, morphological development (villi height and goblet cell number), increased colonisation by beneficial bacteria, and decreased pathogenic bacterial counts. MOS upregulates lysozyme activity and affects oxidative phosphorylation; MOS is involved in immunomodulation of both TLR2b and TLR4 pathways in the ileum and caecal tonsil. The cytokine upregulation of ileal IL-12p35 and IFN-γ in the ileum in MOS treatment implies that MOS supplementation in *C. perfringens*-challenged chickens supports a pro-inflammatory effect, via T-helper type-1 cell-associated pathways, in order to control early stages of infection. In turkeys, MOS may help to reduce the pro-inflammatory response and susceptibility of the colonic epithelium to bacterial binding.

Supplementation of poultry diets YCW (22 studies) improves performance and increases the height and width of the villi, which improve the absorption of nutrients. YCW may enhance the cell-mediated immune response in broiler chickens by modulating the production of cytokines. IL-1β and IL-6 expressions were significantly enhanced in the spleen of YCW-fed birds, suggesting that YCW might act as an immunoprotective agent by up-regulating the inflammatory response leading to enhanced protection against pathogens. Supplementation of broiler diets with yeast also resulted in both local and systemic immune responses, where predominantly TLR2 was involved locally and only TLR4 was involved systemically, with the production of both pro- and anti-inflammatory cytokines.

β-glucan (19 studies) had been shown to increase the size of the primary and secondary lymphoid organs in broilers. β-Glucan acts as an immunoprotective agent by upregulating the inflammatory response, leading to enhanced protection against intracellular pathogens.

·-Glucan also increases pro-inflamatory response (IL-1, IL-2, IFN-γ) and stimulates the Gut Associated Lymphoid Tissue (GALT) to secrete more sIgA in order to enhance mucosal immunological function. The percentage of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ double positive lymphocytes in the intestinal intraepithelial leukocytes was increased in β-glucan supplemented chicks.

Table 17: Mode of action of different prebiotics in poultry

Poultry - Prebiotic	Number of articles	Mode of action
Mannan oligosaccharide (MOS)	**48** Refs: 8360 356 10723 10724 10722 10721 497 1164 1721 10404 11425 12700 12731 4516 7220 4517 7977 9063 2080 10008 4518 1161 8119 7898 7737 7661 5330 3707 46 3561 11152 4865 5384 3002 843 242 724 8359 241 378 9392 10556	MOS increased BW and improved feed conversion. MOS imparted improved intestinal health benefits over antibiotics, as measured by increased morphological development (villi height and goblet cell number), increased colonisation by beneficial bacteria, and decreased pathogenic bacterial counts. MOS significantly up-regulated the transcription of lysozyme (LYZ), which is involved in mucosal immunity. Lysozyme may act as a first defence against bacteria through its hydrolase activity, which breaks down β-1, 4 linkages in the peptidoglycan layer of bacterial cell walls, or through a catalytically independent antimicrobial function. There was a significant association between MOS and several mitochondrial pathways, including oxidative phosphorylation, oxidative stress response, and mitochondrial dysfunction. Oxidative phosphorylation is a pathway that uses energy released by the oxidation of nutrients to produce ATP. Increased expression of these genes suggests that the energy production in the gut cells was increased in birds fed with MOS. Supplementation of MOS in *C. perfringens*-infected birds resulted in an upregulated ileal TLR2b, and ileal and caecal tonsil TLR4 expression, whereas it downregulated caecal tonsil TLR2b expression, suggesting that MOS is involved in the immunomodulation of both TLR2b and TLR4 pathways in the ileum and caecal tonsil. The cytokine upregulation of ileal IL-12p35 and IFN-γ in the ileum in MOS treatment implies that MOS supplementation in *C. perfringens*-challenged chickens supports a pro-inflammatory effect via T-helper type-1 cell-associated pathways to control early stages of the infection. Plasma immunoglobulins were not affected, but the heterophil: lymphocyte ratio, and basophil levels, was significantly affected by prebiotics. Prebiotics did not affect the percentage of T cell or macrophage phenotypes compared with the control birds. MOS or probiotic supplementation to diets of turkey may elevate IgG and IgM levels but reduce the blood T-lymphocyte ratio in turkeys. In addition, compared with MOS, probiotics are more effective in turkeys with regard to inducing humoral immunity. MOS or probiotics may help to reduce the pro-inflammatory response and associated depression in feed intake and growth. MOS alters the bacterial community structure in the turkey caecum. MOS fed to turkeys significantly altered susceptibility of the colonic epithelium to bacterial binding, leading to an altered composition in the enteric microflora closely adjacent to the intestine.

	9393 10252 1621 2081 12242 5336	
Yeast cell wall (YCW)	**22** Refs: 12910 10377 1201 3732 4839 3576 7559 4840 10378 6525 842 12874 10556 7518 12732 4181 402 3562 3983 10740 10587 1408	Supplementation of YCW significantly improved daily weight gain (DWG) during the beginning, ending and overall periods compared with the control birds, but had no effects on feed conversion ratio. Hydrolysed yeast and YCW increased the height and width of the villi in the jejunum, where maximum absorption of nutrients takes place. Longer villi indicate more mature epithelia and enhanced absorptive function due to increased surface area of the villus. It is possible that increased mucin secretion from the goblet cells under the influence of the hydrolysed yeast and the YCW supplementation, coupled with the intrinsic properties of the yeast cell wall mannans binding to bacteria, decreased the number of free bacteria in the lumen and increased their counts on the mucosa. YCW may enhance the cell-mediated immune response in broiler chickens by modulating the production of cytokines. IL-1β and IL-6 expressions were significantly enhanced in the spleen of YCW-fed birds, suggesting that YCW might act as an immunoprotective agent by up-regulating the inflammatory response leading to enhanced protection against pathogens. Supplementation of broiler diets with yeast-derived macromolecules (YDM) resulted in both local and systemic immune responses where TLR2 was primarily involved locally, whereas only TLR4 was involved systemically with the production of both pro- and anti-inflammatory cytokines. The TLR-21 was not observed to be a major receptor involved as a result of YDM supplementation. Supplementation of YDM supports both a pro- and anti-inflammatory cytokine effect via Th1 and Th2 cell-associated pathways, both locally and systemically, with expressions of IL-2, IL-1β, IL-6, IL-8, IFN-β, TGF-β4, IL-10, IL-4, and CD40 locally and IL-12p35, IFN-γ, TGF-β4, and IL-4 systemically. Supplementation of whole yeast cell products, in the absence of any pathogen challenge, increases the IL-10 mRNA amount and Treg population in the caecal tonsils of birds. In the absence of infection, higher IL-10 production and Treg numbers in the gut will facilitate immune tolerance. In the presence of pathogenic bacteria, an inflammatory response mediated by Th17 cells predominates and Tregs lose their suppressive properties and IL-10 production. Supplementing the whole yeast cell wall product decreased coccidial infection, induced increase in *E. coli* and *Salmonella* colonisation and improved IFN-γ mRNA concentration post-coccidial infection. Cell-mediated immune responses are thought to be the most important factors for protection against coccidiosis. CD4+ and CD8+ T-cells populations limit coccidial replication in the intestinal tract. It is possible to hypothesise that the reduction in *Eimeria spp*. replication in the intestine was caused by the YCW-induction of T-cell responses.
Glucan	**19** Refs: 5815 12935 9047 1798 2130 2131 3974 12876 8936	Supplementation of β-glucan did not affect growth performance or blood profiles. β-glucan had been shown to increase the size of the primary and secondary lymphoid organs in broilers. β-glucan may enhance some cell-mediated immune responses of chickens by modulating macrophage ability. β-glucans modulate cytokine profiles, resulting in an enhanced innate and Th1-mediated immune response during an *Eimeria* infection. β-Glucan acts as an immunoprotective agent by upregulating the inflammatory response, leading to enhanced protection against intracellular pathogens. β-glucan exposure increased nitrite and IL-1 production as well as inducing macrophage to proliferate in culture. However, IL-6 production was unaffected.

	1743 1861 3002 2897 9392 9393 154 8935 7688 7893	IL-1, IL-2, IFN-γ and TNF-α may act as signal-messengers in the immuno-regulating network. Levels of these molecules changed according to dosage, corresponding to the levels of β-1,3/1,6-glucan supplementation. As a result of these changes in concentration, the signal that is transferred to the effecting molecule-immunoglobulins, or to the immuno-organs and tissues, is also altered. β-1,3/1,6-glucan acted as both a stimulating and effecting molecule in the regulatory cycle of the macrophage. The levels of IFN-γ and IL-2 may also be actively influenced by feedback from the macrophages. β-1,3/1,6-glucan stimulates the gut associated lymphoid tissue (GALT) to secrete more sIgA in order to enhance mucosal immunological function. The binding of β-glucan to the receptor may cause macrophage, lymphocyte or other blood cells to secrete greater quantities of lower signal molecules. Accordingly, sIgA production might be enhanced in this manner and secreted by the intestinal macrophages and lamina propria lymphocytes. Intestinal sIgA secretion can also be determined by measuring the levels of the cytokines, IL-2, and IFN-γ. Dietary β-glucan supplementation increased the macrophage phagocytic activity, anti-sheep red blood cells antibody response post-boost, and the Phytohemagglutinin (PHA)-P-mediated lymphoproliferative response measured as a toe-web swelling. The percentage of CD4+, CD8+, and CD4+/CD8+ double positive lymphocytes in the intestinal intraepithelial leukocytes was increased in β-glucan supplemented chicks. The primary and secondary lymphoid organs, such as the bursa of Fabricius, thymus and spleen, were larger in β-glucan-supplemented chicks as compared to the chicks fed the basal diet.
Fructooligosaccharide (FOS)	**13** Refs: 2910 4865 5384 442 3002 4111 843 241 4181 402 3562 2683 1408	FOS was comparable with avilamycin (antibiotic) in improving productivity in broilers. It is not clear whether or notplasma immunoglobulins were affected by the prebiotic, but the heterophil:lymphocyteratio (H:L), basophil level, and microbial population in the ileum were significantly affected. Prebiotic FOS-inulin improved the ability of HD11 cells to clear Salmonella Enteritidis by preventing IL-1- associated macrophage cell death. Prebiotic treatment did not influence the NO production, thus suggesting that the FOS-inulin-mediated bacterial clearance was not mediated by NO.
Inulin	**10** Refs: 7581 407 402 442 2897 843 8354 3562 4181 1408	Dietary chicory rich in inulin content can exert beneficial effects on the gastrointestinal tract of broiler chickens as a result of alteration in intestinal architecture and absorption of nutrients. Chicory supplementation seems to influence transport properties of intestinal epithelium. Inulin supplementation significantly increased WBCs and feed conversion ratio (FCR) at the end of grower and finisher periods. Heterophils and H:L ratios were significantly increased but lymphocytes decreased at 42 d of age, when the diets were supplemented with inulin. Addition of inulin to the diet may inhibit the nutritional stress or any stress which causes an increase in lymphocytes ratio because the stress can stimulate the adrenal gland to produce hormones such as estrones, which have a direct effect on lymphatic cells. Inulin seems to have neither a direct nor indirect effect on the blood total protein, albumin, or globulin. The mechanism by which polysaccharides, such as inulin or alginate, impact immunity still require further investigation. It is possible that they directly interact with the macrophages and dendritic cells underlying the gut mucosa, activating the NF-κB pathway through the macrophage receptors (toll-like receptors). Prebiotic FOS-inulin improved the ability of HD11 cells to clear *Salmonella Enteritidis* by preventing IL-1- associated macrophage cell death. Prebiotic treatment did not influence the nitric oxide (NO) production, thus suggesting that the FOS-inulin-mediated bacterial clearance was not

		mediated by NO.
Chitooligosaccharide (COS)	**4** Refs: 4864 6242 12144 4580	Dietary supplementation of chitooligosaccharide appeared to improve the immunity of broilers by promoting the weight of the main immune organs, increasing immunoglobulin M (IgM) secretion, and stimulating macrophages to release TNF-α, IL-1, IL-6, and IFN-β and activating inducible nitric oxide synthase (iNOS) to induce NO. CNP-Cu (chitosan nanopaticle + copper) increased the average daily gain and the contents of IgA, IgG, IgM, complement C3, and complement C4. Thymus, spleen, the bursa of Fabricius indices and the populations of *Lactobacillus* and *Bifidobacterium* in caecal digesta were increased. The increase in serum levels of IgA, IgM, and IgG in response to dietary COS supplementation results from a change in the cytokine-mediated microenvironment. IL-6 induces the final maturation of B-cells into immunoglobulin-secreting plasma cells. Chitosan enhanced IL-6 expression in peripheral mononuclear cells.
Inactivated bacteria-yeast	**3** Refs: 735 12661 12894	Dietary supplementation of yeast autolysate was an effective feed additive in broiler feeding because of increased growth performance, increased immunocompetence, and the reduction of *E. coli* colonisation in the intestine. The use of heat-killed *M. phlei* played a beneficial role as an immunostimulant against caecal coccidiosis in broiler chickens (high body weight gain, reduction of caecal lesion score produced by *E. Tenella*, and increase in IgA concentration). Heat-inactivated *Lactobacillus salivarius, Clostridium butyricum, Bacillus subtilis* mixture could enhance the immune system (increasing antibody titers).
Galactoglucomannan oligosaccharide-arabinoxylan complex (GGMO-AX)	**3** Refs: 3001 3002 3000	Supplementing GGMO-AX consistently resulted in the greatest fold change in pro-inflammatory cytokine expression while inhibiting anti-inflammatory cytokine expression, which indicates a more robust innate immune response. Despite decreasing performance, dietary GGMO-AX improved select fermentation indices as well as the innate intestinal immune response to an acute infection.
Galactooligosaccharide (GOS)	**2** Refs: 5087 4111	GOS has important prebiotic effects, as demonstrated by increases in the beneficial bacteria population in broiler chickens. GOS has a protective effect for alterations in villi length and crypt depth.
Xylooligosaccharide (XOS)	**2** Refs: 12957 2117	XOS selectively stimulate the presence of bifidobacteria in the caeca of chickens. The addition of XOS to feed can increase growth performance, enhance endocrine metabolism, and improve immune function in broiler chickens.
Transgalactooligosaccharide (TOS)	**1** Refs: 843	TOS is supplemented in poultry diets as a mixture with other prebiotics. Therefore, a specific mode of action could not be attributed to it.
Levan	**1** Refs: 12945	Levan (inulin-type fructans) supplementation improves later stage growth performance, increases caecal *Lactobacillus* and *Bifidobacteria* concentrations, and decreases caecal *E. coli* and *C. perfringens* concentrations as well as the NH3 emissions by broilers.
Arabinoxylan	**1** Refs: 2117	Arabinoxylan oligosaccharides (AXOS) selectively stimulate the presence of bifidobacteria in the caeca of chickens.
Mannobiose	**1** Refs: 4688	The upregulation of BLB-1, BF-2, IRF-1, IRF-7, TNFSF15, and TLR3 expression were observed, along with the significant upregulation of several genes involved in immune response and host defence; this suggest that mannobiose (MNB) administration may exert a combination effect on the modulation of the intestinal immune system. An increased production

	of IgA was also observed.

3.4.2.3 Poultry – plant extracts

The systematic review reported 109 studies of plant extracts in poultry. Most of these explored *Thymus vulgaris* derivatives (nine studies), *Allium* derivatives (six studies), carvacrol (six studies), *Curcuma longa* derivatives (six studies), *Astragalus* derivatives (five studies), *Origanum* derivatives (five studies), and other minor groups (Table 18). A large number of substances appeared in less than three papers.

In chickens, thyme oil (nine studies) makes the intestinal barrier stronger against toxic feed-derived substances or endogenously produced toxic metabolites, thus impeding their absorption into the organism. Thyme oil also influences the various antioxidant parameters, but the exact mechanism is unclear.

Garlic (6 studies) increases the relative weight of immune organs in chickens, which causes enhanced lymphocyte proliferation and white blood cell (WBC) counts. Additionally, garlic has potential antioxidative and antimicrobial properties.

Supplementation of diets with carvacrol (six studies) can improve intestinal morphology (villus length and villus height:crypt depth ratio (VH:CD)) as well as cellular and humoral immune response. Carvacrol can also reduce lipid oxidation in broiler meat.

Chickens fed with *Curcuma longa* (six studies) showed an increased humoral and cell-mediated immune response. *C. longa* induces an anti-inflammatory response, which ameliorates the inflammation produced by infection caused by *Eimeria*.

Table 18: Mode of action of different plant extracts in poultry.

Poultry – Plant extract	Number of articles	Mode of action
Thymus vulgaris derivatives	**9** Refs: 1161 1165 10386	Thyme oil makes the intestinal barrier stronger against toxic feed-derived substances or endogenously produced toxic metabolites, thus impeding their absorption into the organism. A thiol redox system is very important in this and consists of the glutathione as well as of thioredoxin system. Thyme oil reduced the level of DNA damage induced by hydrogen

	4502 9394 4173 8571 8572 8814	peroxide (H_2O_2), which is associated with its antioxidant activity. The exact mechanism by which thyme oil influences the various antioxidant parameters is currently unclear. It is possible that the antioxidant properties of thyme oil are being utilised by the cells, thus sparing the intracellular antioxidant systems. It can only be speculated that the different terpene compounds in the oil can modify Kerlch-like ECH-associating protein 1 (Keap 1) at sensor – SH groups through chemical reactions. An increase in IgA concentration in the duodenal mucosa likely reflects the active synthesis of antibodies by the chickens' own immune system. Although the mechanism by which dietary additives can enhance the immune response has not been resolved, several reports have demonstrated the activation of toll-like receptors, luminal captation by dendritic cells, or the stimulation of epithelial cells and the release of pro-inflammatory cytokines.
Allium derivatives	**6** Refs: 8814 10386 8355 5380 4108 11424	Garlic had a direct stimulatory effect on immune cells. The increases in the relative weights of the spleen and thymus of garlic-treated chickens are likely attributed to the enhanced lymphocyte proliferation and the increase in WBC counts. The increase of the spleen, thymus and bursa weight might be understood as connected to the increase in the CD4- CD8- cell ratios. The antioxidative activity of garlic might have protected mature and/or immature lymphocytes from oxidative stress. Additionally, garlic has potential broad antimicrobial activities.
Carvacrol	**6** Refs: 6312 1161 1165 4938 6142 4173	In broiler meat, feed supplement with carvacrol was found to delay lipid oxidation after five and ten days of storage. Carvacrol inhibited the linoleic acid peroxidation by down-regulating the expression of oxidative stress markers, such as cytochrome (CYP)1A1. In these articles, carvacol is primarily used as part of a mixture of essential oils. Therefore, its mode of action on the immune system could not be individually described. The supplementation of diets with these essential oils improved pathological signs (increase in villus length and VH:CD) and increased the cellular and humoral immune responses which could explain the improvement of performance.
Curcuma longa derivatives	**6** Refs: 6143 9394 5378 6142 13096 5078	Chickens fed with *C. longa* showed an increased humoral immune response, as measured by higher serum anti-EtMIC2 antibody. Birds fed the *C. longa* also showed greater cell-mediated immunity, as demonstrated by enhanced spleen cell proliferation response to Con A compared with the non- supplemented diets. Because invasion and intracellular development stages of *Eimeria* parasites in the gut are associated with the induction of local inflammatory response, stimulation of anti-inflammatory response by dietary *C. longa* would lead to decreased gut damage. It is tempting to speculate that this dual mode of action of *C. longa* may serve not only to limit *Eimeria* pathogenicity *in vivo* but also to minimise bystander inflammatory damage to host tissues. In healthy birds, there were no significant differences in the gizzard, heart, spleen, thymus gland, and bursa of Fabricius relative weight (% BW) by the dietary inclusion of *Curcuma longa*. However, serum globulin concentrations were significantly higher, suggesting that dietary inclusion of curcuma improved the health status of broilers.
Astragalus derivatives	**5** Refs: 3967 6267 1732 3966 3968	*Astragalus* polysaccharide (APS) resulted in a beneficial modulation of the microbiota, as evidenced by the significant increase in the concentrations of beneficial bacteria numbers (*Lactobacilli* and *Bifidobacteria*) and decreases in the concentrations of harmful bacteria numbers (*E. coli*). Supplementation of the polysaccharide extracts resulted in enhancement of both cellular and humoral immune responses in *E. tenella*-infected chickens.
Origanum derivatives	**5**	The effects of supplementing diets with essential oregano oil are

	Refs: 6525 1164 522 1161 1165	inconclusive with regard to bird performance. The downregulation of Lipopolysaccharide-Induced TNF Factor (LITAF), IFN-γ, TLR-4 and IL-10 genes by Orego-Stim suggests that it exerted an anti-inflammatory effect on the birds. Additionally, supplementation with essential oils from oregano increases jejunal villus height, which improves the mucosal membrane.
Cinnamon derivatives	**4** Refs: 11424 9394 6142 6312	*Cinnamon* and garlic antimicrobial substances may inhibit intestinal pathogenic organisms and improve digestion and absorption. Cinnamon derivatives are supplemented in poultry dietsas mixtures with other plant extracts. There isclear evidence that plant-derived phytochemicals possess immune-enhancing properties in chickens: induction of lymphocyte proliferation, alteration of intestinal cytokine transcript levels, and increase in the percentage of peripheral blood lymphocytes (PBL) subpopulations.
Nigella sativa derivatives	**4** Refs: 3561 46 11423 5333	Diets supplemented with black seed improved antibody-mediated immunity, increased red blood cell count, haemoglobin concentration and haematocrit percentage. The desirable influence of black seed on haematology can be attributed to the presence of highly active components, particularly thymoquinone and thymohydoquinone, which possess strong antioxidant activities and increase RBC count.
Capsicum derivatives	**3** Refs: 6143 6312 6142	*Capsicum* derivatives are supplemented in poultry diets as mixtures with other plant extracts. There is clear evidence that plant-derived phytochemicals possess immune-enhancing properties in chickens: induction of lymphocyte proliferation, alteration of intestinal cytokine transcript levels, and increase in the percentage of PBL subpopulations.
Echinacea	**3** Refs: 3982 947 8814	*Echinacea* extract supplementation for layer chicks appears not to benefit growth performance and intestinal histology during the growth period. *Echinacea* juice has immune stimulating effects in layers. It seems that the repeated 2 day-trickle stimulation is sufficient to increase immune response.
Soybean derivatives	**2** Refs: 4966 6234	Soybean peptides could increase the density of iIEL and IgA-forming cells in the broiler's intestine. The relative spleen weight in the fermented soybean group was significantly higher than in the control group.
Other Chinese herbs	**2** **Refs: 6279 12887**	DBT (Dangguibuxue Tang) markedly induced the production of IL-2 and IL- 6. DBT not only significantly decreased feed:gain ratios and mortality but also enhanced cellular modulator immune and humoral immune responses in immune-suppressive chicks. Dietary supplementation with FSE (*Forsythia suspense*), BE (berberine), or both can improve growth performance under high stocking density conditions, potentially by increasing the ability of free radical scavenging, reducing oxidative stress, and increasing bursa weight and intestinal levels of healthy microbiota.
Sugar cane	**2** Refs: 12645 410	Some reports indicate that sugar cane extract (SCE) has growth-promoting, immunostimulating, anti-stress effects and antioxidant activity. Increased recovery of the BW, villi, and cells were observed in the SCE groups when compared with the control group.
Syzygium aromaticum	**2** Refs: 13154 3433	Clove oil improves the growth performance of broiler chickens. An increase in the number of IEL and density of cells in the lamina propria were observed in the clove treated group. The interactive effects of clove bud bioactive components and the highly polyunsaturated fatty acids (HUFA) may improve the performance of

		laying hens by fortifying mucosal and systemic immune functions as well as the health indices of the intestinal absorptive area.
Artemisia derivatives	**2** Refs: 5371 5333	LFA (*Lactobacillus*-fermented *Artemisia princeps*) improves growth performance and meat lipid stability in birds. Dietary LFA has probiotic potential by increasing *Lactobacillus spp*. concentrations and decreasing *Salmonella spp*. concentrations in the gastrointestinal tract of birds. *Artemisia* significantly increased monocytes but had no effect on gastrointestinal pH, antibody response, or the relative weight and length of different parts of the carcass. Inclusion of *Artemisia* leaves had a positive effect on gut health and could improve broiler growth performance.
Cuminum cyminum	**2** Refs: 46 3707	Cumin seed does not significantly affect performance and relative organ weight, but its addition to feed may influence white blood cells differentiation.
Ginkgo biloba	**2** Refs: 12920 12918	Supplementation of *Aspergillus* niger-fermented-*Ginkgo biloba* leaves (FG), in the starter and grower diets respectively, improved feed efficiency, intestinal morphology, digestion, and the absorption function of broilers. The FG supplementation reduced IFN-γ expression levels in LPS-challenged birds. The downregulation of both cytokines IL-4 and IL-13 may be part of a homeostatic mechanism of flavonoids and polysaccharides of FG for the maintenance of Th1/Th2 balance in response to extracellular pathogens. Dietary FG was favourable for chickens, especially in the presence of stress, which can be partially ascribed to the immunomodulatory effect of flavonoids and polysaccharides of FG on maintaining Th1/Th2 balance in response to extracellular pathogens.
Carotenoids	**2** Refs: 12277 8368	Elevated plasma carotenoid levels resulted in increased deposition of these compounds into eggs.
Laurus nobilis	**2** Refs: 1161 1165	*Laurus nobillis* is supplemented in poultry diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Humulus	**2** Refs: 1164 9390	Hop extract provided a significant improvement in body weight gain of broiler chickens.
Prunus derivatives	**2** Refs: 6144 5078	Plum promotes protective immunity against coccidiosis as assessed by reduced body weight loss, decreased oocyst shedding, enhanced splenocyte proliferation, and elevated expression of transcripts encoding IFN-γ and IL-15. Continuous ingestion of Probiotic Fermented Four-Herb Combination (*Curcuma longa, Houttuynia cordata, Prunus mume* and *Rubus coreanus*) markedly increased lysozyme activity in serum and the spleen, peripheral blood mononuclear cell (PBMC) proliferation, the CD4+:CD8+ T-lymphocyte ratio in the spleen, and antibody production level in broiler chicks.
Yucca Schidigera	**2** Refs: 839 3983	The height of the jejunal and ileal villi was greater in the yucca extract group. Yucca extract supplementation for layer chicks is beneficial for growth performance and intestinal histology during the 1-60 d growing period.
Achyranthes derivatives	**1**	*Achyranthan* polysaccharides increased splenocyte proliferation, NO, and

	Refs: 1732	IL-2 production in a dose-dependent manner in vitro. Feeding *Achyranthan* polysaccharides significantly increased micro-haemagglutination inhibition antibody titers, bursa of Fabricius index, serum albumin, serum calcium, and nitric oxide (NO) concentrations.
Glycyrrhiza	**1** Refs: 10252	The addition of liquorice extract to broiler diets may reduce abdominal fat content, serum cholesterol, and low density lipoproteins (LDL) concentrations as compared to the control group. Dietary liquorice extract supplementation did not have any negative effects on the body weight or FCR of broiler chickens.
Chicory	**1** Refs: 11947	Raftifeed (inulin from chicory root) increased the number of *Lactobacilli* in the ileum and caused reduction in coliform counts in the ileal and caecal digesta.
Citrus by product	**1** Refs: 1165	*Citrus* peel oil is supplemented in poultry diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Zingiber officinale	**1** Refs: 4938	Ginger oil is supplemented in poultry diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Bran derivatives	**1** Refs: 10065	Modified Arabinoxylan Rice Bran (MGN-3) stimulates the T-cell immune system in the spleen of chickens. The levels of cluster of differentiation 3 (CD3), IL-2, and IFN-γ mRNA in the spleen of chickens increased with the supplementation of MGN-3 in the diet. Mitogen induced proliferation of splenic mononuclear cells (MNC) and blood MNC phagocytosis in chickens fed MGN-3-supplemented diets was significantly greater than in the control group.
Pinus derivatives	**1** Refs: 8214	The addition of pitamin to the diet of broiler chickens increased the growth of *Bifidobacterium* and *Lactobacillus* species, reduced the numbers of *Escherichia coli* and *Salmonella* among caecal microorganisms, and improved the growth of the cells of major immune organs, including the spleen, the thymus, and the bursa of Fabricius; it also boosted the immunoglobulin IgG content.
Propolis	**1** Refs: 378	Propolis is adequate to enhance the productive performance of broiler chickens.
Tannin	**1** Refs: 6788	Dietary tannins not only affect the proper performance of the chickens, due to their anti-nutritional characteristics, but may also reduce the efficacy of anti-coccidial vaccines.
Medicago sativa	**1** Refs: 2683	In alfalfa molt diets, the concentrations of lactic acid were greater than they were in the control group.
Zea mays	**1** Refs: 12598	The proportion of CD3+CD4+ lymphocytes of intestinal mucosal lymphocytes (IMLs) in chickens fed fish-oil diets on 21 d and 42 d of age was significantly higher than those in chickens fed corn oil. The proportion of intestinal CD3+CD8+ lymphocytes of chickens fed fish oil diets was significantly lower than those of chickens fed corn oil. Fish oil consumption enhanced IL-2 secretion of IMLs, but corn oil consumption decreased IL-2 secretion. Corn oil increased the mRNA abundance of cGRP, cAMP level and adenylyl cyclise bioactivity.
Carthamus	**1** Refs: 6139	Increased splenic lymphocyte proliferation, in addition to increased percentages of CD4+ T cells and decreased CD8+ cells, was observed in animals fed a safflower-supplemented diet. IFN-γ, IL-8, IL-15 and IL-17 transcripts in the safflower-supplemented group were higher than they were in the non-supplemented controls.

Solanum tuberosum	**1** Refs: 4107	Purple sweet potato increased antibodies against vaccines and augmented the proliferations of splenocytes and thymocytes.
Camellia sinensis	**1** Refs: 5333	*Camellia* increased gizzard and proventriculus pH, villi length, and crypt depth but decreased primary antibody response, total white blood cell count, and cholesterol concentration.
Mentha piperita	**1** Refs: 2910	Peppermint essential oil increased duodenal crypt depth. This study illustrated that Peppermint oil could not be suggested as an effective alternative for antibiotics.
Panax ginseng	**1** Refs: 240	Red ginseng extract administration improved the lymphocyte level while exerting no influence on RBC or WBC.
Rubus coreanus	**1** Refs: 5078	Rubus coreanus is supplemented in poultry diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Sanguinaria canadensis	**1** Refs: 7650	The villi height of duodenum and jejunum in the medicinal plant treated groups was improved. Immune response was significantly higher. Supplementation of plant extract (Sangrovit®) to broiler feed may reduce the incidence of *Campylobacter* infection in these birds.
Achillea	**1** Refs: 12666	The administration of yarrow can reduce the levels of serum lipids and can boost the secondary immune response in broilers. Moreover, its administrationled to a reduced pathogenic bacteria population in the gastrointestinal tract (GIT), which could help improve intestinal health
Anethole	**1** Refs: 5379	Broiler chickens continuously fed from hatch with an anethole-supplemented diet and orally challenged with live *E. acervulina* oocysts showed enhanced BW gain, decreased faecal oocyst excretion, and greater *E. acervulina* profilin antibody responses compared with infected chickens fed an unsupplemented standard diet. The levels of transcripts encoding the immune mediators IL-6, IL-8, IL-10, and the tumour necrosis factor ligand superfamily member 15 (TNFSF15) in intestinal lymphocytes were increased in *E. acervulina*-infected chickens fed the anethole-containing diet, compared with untreated controls.
Canavalia ensiformis	**1** Refs: 7145	The data indicated that Con A binds to the cells of the gastrointestinal tract, passes into the general circulation and, eventually, elicits an immunological response without affecting the production of antibodies to *Brucella Abortus*.
Myrtus communis	**1** Refs: 1165	*Myrtus communis* is supplemented in poultry diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Foeniculum vulgare	**1** Refs: 1165	*Foeniculum vulgare* is supplemented in poultry diets as a mixture with other essential oils. Therefore, a specific mode of action could not be attributed to it.
Gossypium	**1** Refs: 11219	In solid-state fermented cottonseed meal group, the concentration of serum immunoglobin M, as well as the content of complements (C3, C4), was greater than those in the control group.
Isoflavones	**1** Refs: 5151	Genistein and hesperidin have the potential to improve immunity, and these also develop the morphometric structures of the small intestine in a dose-dependent manner. Moreover, both compounds were found to be effective in attenuating LPS-induced deterioration of intestinal architecture.
Cocos nucifera	**1**	Mannanase-hydrolysed copra meal might be effective for activating

	Refs: 4687	intestinal absorptive function, and this functional activation promotes the growth of the chickens.
Silybum marianum	**1** Refs: 1652	Milk thistle may effectively stimulate the immune function and growth performance in the presence of immunosuppressant aflatoxin B1 in the feed.
Piper nigrum	**1** Refs: 13096	Dietary supplementation with black pepper enhanced the performance and health status of broiler chickens.
Coriandrum Sativum	**1** Refs: 13096	Dietary supplementation with *Coriandrum Sativum* enhanced the performance and health status of broiler chickens.
Epimedium derivatives	**1** Refs: 1773	Oral administration of EFO (epimedium polysaccharide (EP)-propolis flavonoid (PF) oral liquid) to chickens that had received the Newcastle Disease vaccine significantly promoted secretion of sIgA and IL-17 from the duodenum and the jejunum and increased the numbers of IELs in the duodenum mucosa and IgA+ cells in the jejunum mucosa and the cecum tonsil.
Houttuynia cordata	**1** Refs: 5078	*Houttuyna cordata* is supplemented in poultry diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Acacia derivatives	**1** Refs: 11947	Supplementation with water-soluble carbohydrates from *Acacia senegal* was not effective in controlling necrotic enteritis (NE). Feeding of *Acacia* extract caused reduction in coliform counts in the ileal and caecal digesta. The *Lactobacillus* count in the same group was significantly higher.
Arthropodium cirratum	**1** Refs: 11947	Supplementation with water-soluble carbohydrates from lili extract was not effective in controlling NE. Feeding of lili extract caused reduction in coliform counts in the ileal and caecal digesta. The *Lactobacillus* count in the same group was significantly higher.
Ilex paraguarensis	**1** Refs: 3747	Yerba mate promoted antimicrobial activity against foodborne pathogens and enhanced the growth of LAB in vitro, but in vivo yerba mate did not decrease *Salmonella Enteritidis* colonisation.

3.4.2.4 Poultry – animal by-product

There were a few studies on animal by-product substances (10 studies), such as lactoferrin (3 studies)) (Table 19). Chickens fed lactoferrin showed an increase in serum IgA and IgG as well as enhanced expression of IFN-γ and IL-12 in T-lymphocytes.

Table 19: Mode of action of animal by-products in poultry

Poultry – animal by-product	Number of articles	Mode of action
Other	**4** Refs: 6415	**Rabbit sacculus rotundus antimicrobial peptides (RSRP):** RSRP could effectively enhance the height of the intestinal villus of the duodenum and jejunum. Significantly increased number of intestinal IEL (iIEL) and area of IgA-secreting cells in the duodenum, jejunum, and ileum of RSRP group were identified.

	6396 7692 8935	**Squid Ink:** Squid ink could improve growth performance, antioxidant ability, and immune function (relative weight of immune organs) in growing broiler chickens. **Multienzyme complex (carbohydrolases and phytases):** The inclusion of enzymes in conventional or alternative diets reduced the number of CD3 in the mucosa of the ileum. The use of alternative plant feed-stuffs in broiler diets promoted an increase in intestinal lesions and enhanced cellular immune response. The undesirable gut health condition can be mitigated using a multi-enzyme complex.
Lactoferrin	**3** Refs: 3520 4633 4632	pLF (porcine lactoferrin) led to a significant increase in serum IgA, IgG and IBD specific antibody titers. pLF administration enhanced the expression of IFN-γ and IL-12 in chicken T lymphocytes, and pLF enhances cell mediated immunity and augments the ability of IBD vaccination to strengthen subsequent anti-viral response.
Oils	**2** Refs: 12598 3433	The proportion of CD3+ CD4+ lymphocytes of intestinal mucosal lymphocytes (IMLs) in chickens fed fish oil diets was significantly higher than those in chickens fed corn oil and poultry oil diets. The proportion of intestinal CD3+CD8+ lymphocytes of chickens fed fish oil diets was significantly lower than those of chickens fed corn oil and poultry oil diets. Fish oil consumption enhanced IL-2 secretion of IMLs stimulated with concanavalin A. The increased proportion of CD3+CD4+ lymphocytes of gastrointestinal (GI) tract in chickens fed dietary fish oil promoted IL-2 and IL-4 secretion of IMLs. Fish oil decreased the mRNA abundance of calcitonin gene related peptide (cGRP) in intestinal mucosa. The down-regulating cAMP signalling of IMLs in chickens fed dietary fish oil enhances the proportion of CD3+CD4+ T lymphocytes and production of IL-2 of IMLs in chickens fed dietary fish oil. Supplementation of fish oil and clove bud powder, either alone or in combination, markedly decreased the ileal *Escherichia coli* and *Salmonella*counts.
Antibodies	**1** Refs: 6699	Dietary administration of sIgY has the potential to reduce the concentration of sIgA via inhibition of intestinal bacterial proliferation in challenged birds and may also reduce serum IgA concentration as a sIgA-dependent variable. Dietary administration of sIgY could reduce this parameter by direct depression of local pathogens as well as indirectly decreasing inflammatory reactions induced by serum IgA. The reduction of lamina propria lymphatic follicle proliferation, in addition to local effects of sIgY on mucosal surfaces, might be due to the fact that IgY can be taken up by pinocytosis from the intestinal lumen and subsequently transported into the lamina propria and then passed into the lymph fluid without inducing an inflammatory response.

3.4.2.5 Poultry – other substances

The systematic review reported 106 studies of other substances / agents in poultry. Most of these concerned organic acids derivatives / fatty acids (31 studies), vitamins (13 studies), minerals (12 studies), amino acids and derivatives (12 studies), fungi / mushroom (10 studies), enzymes (nine studies) and other minor groups (Table 20). Each group of substances /agents includes several products, each with different modes of action. Due to the complexity of the data, a summary of the mode of action for each group cannot be described within this report.

Table 20: Mode of action of other agent / substances in poultry

Poultry - Other	Number of articles	Mode of action
Organic acids derivatives / Fatty acids	**31** Refs: 2909 7650 4516 4518 2212 6700 11768 11152 218 11772 4938 804 10064 1888 9276 6482 9189 12175 11052 11053 242 7737 8359 8733 12894 10545 10546 12176 10587 377 1408	**Butiric acid (BA)** reduced villus length and increased microvillus and crypt depth. Dietary BA inclusion did not affect the counts of lymphocytes, heterophils, monocytes, basophils and eosinophils. Butyric acid reduces virulence gene expression and invasiveness in *S. enteritidis*; it also decreases in invasion and has been proposed to lead to decreased caecal colonisation. Prebiotic, butyric acid glycerides, and probiotics or their combination with prebiotics or butyric acid glycerides enhance the resistance of birds and partially protect against *Coccidiosis*. The effects of the different SCFA on colonisation with *Salmonella* can be explained by the alteration of *Salmonella* virulence gene expression and invasion of epithelial cells after contact with the respective SCFA. Contact of the bacteria with propionic and butyric acids resulted in a decrease in invasion. Chickens infected with *C. perfringens,* and treated with the combination of sodium butyrate and essential oils, showed significantly better BW gain, increased villus length and villus length: crypt depth ratio as well as decreased gross pathological and histopathological lesion scores compared with the control group. A combination of Cyclic adenosine monophosphate (cAMP) agonists and butyrate (or butyrate analogues) with a strong capacity to induce host defence peptide (HDP) synthesis may have the potential to augment animal innate immunity. **CLA (conjugated linoleic acid)** alleviated the immunosuppression of T-lymphocytes in broiler chickens exposed to cyclosporin A through increasing the peripheral blood T-lymphocyte proliferation and IL-2. The 2 CLA isomers enhanced T-lymphocyte proliferation at low concentrations and inhibited T-lymphocyte proliferation at high concentrations. At a cellular level, the effects of CLA on the alleviation of immunosuppression in T-lymphocytes are mainly attributable to increasing the signalling molecules, such as phospholipase C and protein kinase C. Feeding low dietary ratios of linoleic to linoleic acid to newly hatched chicks modified fatty acid composition of immune tissues, resulting in an increase in antibody production against standard vaccines. The proliferation of lymphocytes depends on the production of IL-2. One may speculate that the effects of dietary alpha-linoleic acid (LNA) on lymphocyte stimulation is caused by the increased competition of n-3 and n-6 PUFA for the binding sites of cyclooxygenase to produce Prostaglandin E2(PGE2). **Phenyllactic acid (PLA)** depressed FI and BWG during the first 7 d, but growth performance improved after 21 d. White blood cell, lymphocyte concentration, red blood cell, total protein, and albumin concentrations were higher in the PLA group. An improvement of immune status was detected by densely populated immunocompetent cells in the lamina propria and submucosa of caecal tonsils and ileum and also in the cortex and medulla of bursa follicles in citric acid-supplemented chicks. Combined probiotics (a blend of *Lactobacillus acidophilus, Lactobacillus casei, Streptococcus faecium,* and *Saccharomyces cerevisiae*) and organic acid (a blend of ascorbic and citric acids) supplementation resulted in variable responses of intestinal histomorphology showing increased goblet cell numbers in the ileum of birds treated. The presence of organic acids could enhance pro-inflammatory response. The enhancements in feed conversion ratio due to the use of acetic acid at different concentrations could be attributed to the antimicrobial and buffering capacity of acetic acid. It was found that broilers fed phytase + commercial mixture of organic acids had higher levels of IgG in the primary immune response, and higher total Ig and IgG in the secondary response compared with all other groups, suggesting an additive effect of phytase and organic acid on the immune response to SRBC. When chickens fed a diet deficient in P and supplemented with this blend, the VH, the CD, and the VH/CD ratio were restored. Supplementation with an encapsulated organic acid blend changed the microbial activity in the ileum and caeca to some extent, however without a gross change in the caecal bacterial community structure. Dietary supplementation with 5-aminolevulinic acid (5-ALA) provides mild oxidative stress and enhances the expression of regulatory factors involved in the immune response, resulting in protection from disease and accompanied by a reduction in growth

		performance. Dietary 5-ALA supplementation increases the plasma TBARS concentration and the expression levels of splenic CD3 mRNA in a dose-dependent manner. This implies that dietary 5-ALA supplementation induces oxidative stress and enhances the T-cell system in chickens. In chicken HD11 macrophages and primary monocytes, induction of HDPs (host defence peptides) is largely in an inverse correlation with the aliphatic hydrocarbon chain length of free fatty acids, with SCFAs being the most potent, medium-chain fatty acids moderate and long-chain fatty acids marginal. Additionally, three SCFAs, namely acetate, propionate, and butyrate, exerted a strong synergy in augmenting HDP gene expression in chicken cells. The goblet cell numbers in formic and propionic acid group was significantly increased in all intestinal segments compared to the other groups. Organic acid combination may probably promote the effects of probiotic-prebiotic combination.
Vitamins	**13** Refs: 5815 13087 9546 8936 7489 8119 8921 1881 8935 8174 7519 7520 12998	The combination of high levels of Arg and higher-than-industry levels supplementation of **vitamin E** have an important immunomodulation effect on the cell- and humoral-mediated immune response of broiler chickens by increasing the amount of T- and B-cells and the CD4+ and CD8+ lymphocyte subpopulations. The supplementation of α-tocopherol significantly enhanced the capacity of macrophages to engulf unopsonized SRBC. These macrophages can effectively perform their function by binding, internalizing and degrading foreign particles by lysosomal acid hydrolysis. The enhanced engulfment may be due to potential effects of α-tocopherol on receptors located on the cell membrane for different functions and are Fc portion of immunoglobulins. The increase in the mutagenic activity of T-lymphocytes can be enhanced further by the supplementation of α-tocopherol and has a positive effect on the cell-mediated part of immune response. Vitamin E induces an increase of IgA in the circulation and at the local intestinal site. Both vitamins E and C improved antibody responses to inoculated sheep red blood cells (SRBC) and Newcastle disease virus (NDV) vaccine, lymphocyte proliferation in response to mitogen and activities of antioxidant enzymes red blood cell catalase (RBCC) and glutathione peroxidase (GSH-PX) in layers. Dietary vitamin E was associated with elevation of CD4+, CD8+, as well as CD4+ CD8+ T lymphocytes in Listeria-infected turkeys, when compared with infected turkeys on control diets. Splenic expression of IL-6 was higher in Leghorns fed the basal or ascorbic acid diets, rather than the β-glucan diet, whereas the opposite relationship was observed in the Fayoumi line. The lack of a significant diet effect on IFN-γ supports an increase in T-helper cell number rather than an augmentation of the T-cell-mediated immune response. The addition of immune-enhancing substances to chicken diets may have different effects due to the genetic backgrounds of the lines. When breeders received a high mix (retinol (vitamin A), tocopherol (vitamin E), ascorbic acid (vitamin C), thiamine (vitamin B1), riboflavin (vitamin B2), zinc, copper and selenium) the number of infiltrating polymorphonuclear leukocytes in the intestine was higher compared with those of breeders receiving basal amounts of minerals and vitamins. Additionally, the recovery rate of intestinal lesions, cystic crypts, and villus atrophy, as observed by histopathology, was faster in the groups in which the breeders received high mix. Supplemental 25-OH-D3 (hydroxylation of vitamin D) had no apparent effect on growth performance. However, it resulted in lighter relative weight, longer villus length, and shorter crypt depth of the small intestine. Dietary **Folic Acid** (FA) enhanced expression of IL-8 in the spleen; however, there was a downregulation in the expression of IL-8 in the caecal tonsils, indicating a pleiotropic effect of FA in older laying hens under acute LPS challenge. Interleukin-18 and IL-1β are related pro-inflammatory cytokines critical to the initiation of inflammatory responses. The downregulation of IL-1β and IL-18 in the caecal tonsils may partially explain the beneficial anti-inflammatory effects of FA in young laying hens exposed to an acute LPS challenge. This downregulation reduces the likelihood of an immune response to an antigen and serves to control inflammatory responses once the pathogen has been cleared. The balance between Th1 (IFN-γ) and Th2 (IL-10) inflammatory responses in response to LPS was therefore enhanced.

Minerals	**12** Refs: 5252 7811 8572 11225 12184 11224 12180 7452 8921 12525 4685 4227	Natural **Clinoptilolite** (NCLI) and modified Clinoptilolite (MCLI) improved the permeability of the intestinal mucosa, reduced the extent of intestinal mucosa damage, and ultimately protected intestinal mucosal barrier function. Pre-treatment with NCLI and MCLI significantly decreased the levels of pro-inflammatory cytokines. These data suggested that NCLI and MCLI could decrease the concentrations of TNF-α, IL-1 and IL-10. This indicated that NCLI and MCLI could improve the permeability of the intestinal mucosa and played a protective role in the broiler intestinal barrier function. The addition of **Zinc**-bearing clinoptilolite (ZnCP) to the diet produced a decreasing effect on the *Salmonella* population, suggesting that ZnCP can regulate the caecal microflora of broilers after *Salmonella* infection. The protective effects of ZnCP on growth performance, microflora colonies, and serum diamin oxidase (DAO) of broilers infected by *Salmonella* might attribute to the antibacterial activity and the adsorption of bacteria or toxins onto ZnCP. Therefore, ZnCP could act as an antibacterial agent against *Salmonella*, inhibiting its growth and improving the gut health. Results indicated that supplementation with ZnCP significantly decreased the relative weight of the spleen. It is likely that ZnCP could adsorb *Salmonella* bacteria or toxins and inhibit its activity, decreasing the transportation of *Salmonella* into the spleen and thus reducing the immune reactions against *Salmonella* and spleen weight. When thyme oil and **selenium** are administered together, there is an increase in the antioxidative capacity of broiler chickens' tissues. Dietary inorganic **chromium** (Cr) supplementation as feed additive (2 mg/kg basal diet) has improved the production performance, and potentiated the immune-competence of heat-stressed broiler chickens. The increase in lymphocytes and decrease in heterophils count, with the subsequent reduction of heterophil:lymphocyte ratio of Cr supplemented heat-stressed chickens observed, could be related the Cr-induced reduction in serum cortisol. **Vanadium** in excess diet reduces the ileac T-cell population, the percentage of T-cell subsets, and the contents of cytokines such as IL-2, IL-6, and IFN-γ in the ileum. These changes may affect the immune function of local intestinal mucosa in broilers. The ameliorative effects of dietary **sodium** (Na)-bentonite on the reduced percentage and mean of phagocytosis caused by Aflatoxin could be attributed to the role of Na-bentonite as a sequestering agent against aflatoxin (AF) present in the diet through reducing its bioavailability in the gastrointestinal tract.
Amino acids and derivatives	**12** Refs: 5360 13087 1313 9055 10008 12451 12720 12610 530 434 2872 12907	Use of L-**threonine** increased intestinal morphology parameters such as crypt depth, villi height, and width in both the jejunum and ileum segments. However, villi height and width and crypt depth increased in both jejunum and ileum segments when dietary L-threonine increased. L-threonine increases the concentrations of IgA antibody in the ileum. The addition of L-threonine in the diet resulted in increased levels of IgG, but no difference was found in the concentration of IgG antibody in the ileum or the jejunum. Expression of jejunal and ileal mucin (MUC)-2 mRNA increased in a linear fashion ($P<0.01$) by increasing levels of L-threonine. The combination of high levels of **Arg** and high supplementation of vitamin E have an important immunomodulation effects on the cell- and humoral-mediated immune response of broiler chickens by increasing the amount of T- and B- cells and the CD4+ and CD8+ lymphocyte subpopulations. Increasing L-Arg level in the diet supports immune response due to the production of NO in healthy broilers. Significant improvements in body weight gain were observed when **Gln** was supplemented in the feed. The birds fed diets supplemented with Gln had significantly longer intestinal villi than birds in the control group, indicating increased nutrient absorption. Higher IgA concentrations in the serum, bile, and intestines observed in the birds fed diets supplemented with Gln. IgG levels increase in birds fed diet with Gln. The supplementation of Gln in diets fed to chicks significantly promoted the growth of the spleen and thymus. **Cysteamine** can induce proliferation and differentiation of IgA-positive cells and iIEL in the intestinal mucosa of chickens by reducing the number of somatostatin-positive cells.
Fungi - Mushroom	**10** Refs: 4576 3583	Dietary mushroom supplementation significantly increases the numbers of *Lactobacilli spp.* in ileum and *Lactobacilli spp*. and *Bifidobacteria spp*. in the caecum of chickens. Additionally, it decreases the number of *Salmonella*, *Bacteroides spp.*, *Enterococci*, and *E. coli* populations significantly. Changes in the species present in the ileal or caecal bacterial community, after fermentation of mushroom constituents, may underlie an effective

	3967 11425 12441 12440 3966 3968 2264	mechanism. Dietary mushroom supplementation affected neither intestinal morphology nor the mechanisms at the intestinal level.
Enzymes	**9** Refs: 7692 7977 6389 10407 6408 11818 3442 1161 2909	**Lysozyme** has bacteriostatic and bactericidal activity against many Gram (+) bacteria and only a limited ability to affect Gram (-) bacteria. Enzymatic hydrolysis of lysozyme has been found to enhance its activity by exposing antibacterial portions of the protein and producing antibacerial peptides. Lysozyme reduced *E. coli* and *Lactobacillus* counts in the ileal digesta of birds, suggesting that digestive enzymes and other compounds, such as sIgA in the gut, may have induced the novel antimicrobial activity and broadened the spectrum of activity of lysozyme in vivo. In addition, the apparent effect of lysozyme *on E. coli* and *Lactobacillus* counts could also be indirect in birds challenged with *C. perfringens*; this occurs via its inhibitory effect on *C. Perfringens* colonisation, which can reduce their counts. **Phytase** addition significantly increased the percentages of erythrocyte rosette-forming cells (ERFC) and erythrocyte-antibody complement cells (EAC), indicating that the proliferations of T- and B-cells were induced by factors that may result from the substantial concentrations of lower inositol phosphates created by dietary phytase. Because the bursa is the source organ for B-cells, the development of the bursa may induce the proliferation of B-cells. Thus, the growth-promoting effect of phytase may be expressed via both nutrient release and a physiological regulation mechanism. The percentages of CD4+ and CD8+ T-cells are enhanced by phytase addition, indicating a potential for increased activity of immunocytes. Part of the nutritional improvements associated with phytase may be mediated through improvements in immunocyte activity. Birds fed high-phytate diets returned a lower concentration of sIgA in jejunal mucosa, which may partially result from dilution when mucin is hypersecreted, as stimulated by phytate. The mechanism by which phytate influences mucin integrity is thought to be related to the highly (pH dependent) reactive nature of dietary phytate. When feed is exposed to the low pH conditions in the proximal gut, phytate is solubilised and can react electrostatically with basic amino acid residues in dietary protein. Supplementing diets with phytase restores the VH, CD, and VH:CD when broiler chickens are fed a diet deficient in P. **Xylanase** increases the relative weight of the spleen, suggesting that enzyme supplement accelerated the development of the immune organ. It increases serum antibody titers to NDV, suggesting that enzyme supplement enhanced humoral response. The enzyme supplementation increased lymphocyte proliferation in response to PHA and NK cell activity significantly, suggesting that the cell-mediated responses were enhanced. The addition of the enzyme likely enhanced the digestion of feed and the absorption of nutrients, which in turn could have an effect on body immunity. Additionally supplementation with xylanase reduces *Salmonella* after infection.
Peptides	**5** Refs: 5598 5599 12148 5596 5597	Not only did *Brevibacillus texasporus* (BT) prime the heterophils and monocytes for an increase in transcription of pro-inflammatory cytokines induced by inflammatory agonists, but it also up-regulated expression of inflammatory chemokines mRNA. Although BT priming modulated the expression of cytokine mRNA in the leukocytes stimulated by different inflammatory agonists, BT on its own neither directly induced cell functional activity nor gene expression of either the pro-inflammatory cytokines or inflammatory chemokines. Cytokine gene expression in avian heterophils and monocytes can be regulated by the BT peptides to allow the cells to respond to a stimulus in a qualitative manner. BT peptides appear to servea preventive function in the innate immune cells, which can initiate cell migration to the site of infection, and the increased phagocytosis can kill off the invading bacteria. The increased expression of CXCLi1 and CXCLi2 shows that heterophils and monocytes can direct the recruitment of further innate immune cells that lead to the site of infection, increasing the ability of the host to limit the infection. Accordingly, these results imply that both cell types are proficient in amplifying the local acute inflammatory response. Furthermore, the expression of IL-1 and IL-6 in the BT-primed heterophils and monocytes would further lead to enhanced bacterial clearance. BT, as a feed additive, reduces *Salmonella enteritidis* (SE) caecal colonisation. This

		protection is not the result of direct antibacterial activity of the BT on the SE. In mammals, most of these cationic peptides have little direct antimicrobial activity but do enhance innate immune response without harmful inflammatory responses. Swine Intestinal Antimicrobial Peptides (SGAMP) could increase the number of mast cells, goblet cells, and IEL, suggesting that SGAMP can improve the integrity of intestinal mucosal surface structure. Markedly increased sIgA-producing cells in the duodenum, jejunum suggests that SGAMP could improve mucosal immune responses for an extended period of time. These results document the regulating capabilities of SGAMP in modulating the outcome of intestine immunocompetent cells.
Algae	**3** Refs: 12633 5181 402	**1:** Dietary sAO (Sodium alginate oligosaccharides from brown algae) inclusion at 0.04 and 0.2% of the diet increased the caecal populations of LAB and reduced caecal *Salmonella* colonisation. *Salmonella enteritidis*-specific sIgA levels were significantly increased by dietary sAO. Dietary sAO increased the expression of three cytokines (IFN-γ, IL-10, and IL-1β). **2:** The additives used were dried*Chlorella* powder (DCP), *Chlorella* growth factor (CGF), and 1.0% fresh liquid *Chlorella* (FLC). The number of white blood cells was significantly higher in broilers fed FLC. Dietary supplementation of *Chlorella* significantly increased the plasma IgA concentration of chickens. Plasma IgM concentration was higher in DCP and FLC treatments than in the control group, and plasma IgG concentration was also higher in the FLC treatment compared with other treatments. **3:** *Algae* used in this study is supplemented in poultry diets as a mixture with other substances. Therefore, a specific mode of action could not be attributed to it.
Nucleotides	**1** Refs: 11053	cAMP agonists synergise strongly with butyrate or butyrate analogues in avian β-defensin 9 (AvBD9) induction in macrophages and primary jejunal explants. Oral supplementation of forskolin, an adenylyl cyclase agonist in the form of a *Coleus forskohlii* extract, was found to induce AvBD9 expression in the crop of chickens. The results suggest the potential for concomitant use of butyrate and cAMP signalling activators in enhancing HDP expression, innate immunity, and disease resistance.

3.4.3. Bovine

The most common substances and agents studied, with regard to their effects on immunity in bovines, were probiotics (Table 6).

3.4.3.1 Bovine – probiotics

The systematic review reported 50 studies on probiotics in poultry. Most of these concerned *Lactobacillus spp.* (14 studies), *Enterococcus spp.* (10 studies), *Bacillus spp.* (6 studies), *Saccharomyces spp.* (four studies), and other minor groups (Table 21).

The supplementation of poultry diets with *Lactobacillus spp.* (14 studies) improves animal performance and increases the number of LAB in rumen, duodenum, and jejunum and decreased the prevalence of microrganisms capable of causing inflammation. Acidification capability was increased, as were hydrogen peroxidase and bacteriocins, which might be active against some pathogens. The increased number of peripheral monocytes expressing TLR2 (inducing pro-inflamatory cytokines i.e. IL1b, IL6, TNFα) might be associated with the

formal reaction of innate immunity in response to increasing and changing bacterial diversity in the gastrointestinal tract of calves receiving the probiotic.

Enterococcus spp. administration (10 studies) during the first weeks following birth increases the healthof calves, making them more resilient to subsequent stressful situations. Oral administration of *Enterococcus spp.* in calves may have an immunostimulatory effect, promoting earlier recovery of IgA levels in mucosal immunity and enhancing the intestinal mucosal immunity, increasing the number of IELs and reinforcing the intestinal mucosal barrier against infection.

Table 21: Mode of action of different probiotics in bovine

Bovine - Probiotic	Number of articles	Mode of action
Lactobacillus spp.	**14** Refs: 5273 3302 13145 3301 8737 8736 5416 9229 491 92 4257 5274 2876 12224	Lactic acid bacteria improved BW and FI, increasing the number of LAB and LAB/coliform ratio in rumen, duodenum, and jejunum and decreasing the prevalence of E. coli O157. This effect on E. coli O157 was further supported by a decrease in ileal lamina propria thickness for treated steers, which might indicate that harmful microorganisms capable of causing inflammation were inhibited. *L. animalis* and *L. paracasei* subspp. *paracasei* have indicated an acidification capability due to the production of organic acids (acetic and lactic acid), hydrogen peroxide, and bacteriocins; these antimicrobial-like compounds might be active against some pathogens. *L. acidophilus* with/without *L.plantarum* increase of IgG, haemoglobin (Hb), and packed cell volume (PCV) and decrease in cholesterol (CHLO) and total lipids (TLI) concentration. The feed additive increased expression of IL-4R. Enhanced expression of IL-4R has the potential to increase IL-1β production and to enhance cell death. A possible mechanism by which the additive increases neutrophil IL-1β secretion is via IL-4R-dependent activation of ICE. Increased expression of epidermal growth factor (EGF) receptor implies that the neutrophils of the treated animals may be more sensitive to EGF signalling and therefore may be more sensitive to TNF. Analysis of genes, which were differentially regulated by the feeding OmniGen-AF, indicated that three generalised changes in neutrophil physiology may be induced, including: i) apoptosis, ii) cell-to-cell communication and iii) sensitivity to extracellular signalling (i.e. via altered expression of cytokine/hormone receptors). Mixtures of probiotics containing Lactobacillus increase the number of CD3+CD45R+ T cells, which may indicate a nonspecific but enhanced immune state. Similar to CD3+ cells, increased numbers of CD4+CD45R+ and CD8+CD45R+ T cells are observed. They may be involved in immune system responses, along with direct or indirect immune-stimulatory effects of the probiotic in the calves. The higher number of Workshop cluster 1 (WC1) +γδ T cells in calves receiving the probiotic was associated with an increased number of CD4+ cells. The increased number of peripheral monocytes expressing TLR2 might be associated with the formal reaction of innate immunity in response to increasing and changing bacterial diversity in the gastrointestinal tract of the calves receiving the probiotic. Increased expression of IL-6, IFN-γ and TNF-α in peripheral leukocytes may be dependent on the period after administration and numbers of activated T-lymphocytes or monocytes in the treatment group.
Enterococcus spp.	**10**	Probiotic administration during the first weeks after birth increases the wellness of calves by sustaining their intestinal health and making them more physiologically

	Refs: 6960 11571 8737 8736 9229 491 12954 2914 3569 4895	resilient in facing subsequently stressful rearing conditions. Probiotic supplementation reduced the faecal count of *Clostridia* and *Enterococci*. Oral administration of *Enterococcus* in calves may have an immunostimulatory effect, promoting the earlier recovery of IgA levels in mucosal immunity, enhancing the intestinal mucosal immunity, increasing the number of IELs, and reinforcing of the intestinal mucosal barrier against infection by the hyperplasia of epithelial goblet cells in the small intestine. Animals fed with the probiotic group also showed a higher number of mast cells. Intestinal mucosal mast cells are important immunocompetent cells in the intestinal mucosal immune response, and they exert multifunctional roles. These results supported the idea that the specific effect on the gut defence mechanisms was that *Enterococcus* had an intense influence on the number of mucosal immune cells and that increased intestinal mucosal immune cells can enhance the defence system of the body. Therefore, diarrhoea, mortality, and morbidity will be decreased. However, supplementation with *E. faecium* to feedlot steers under high-grain diet for a period of 11 d had no effects on acute phase proteins measured (i.e., serum amyloid A (SAA), LBP, haptoglobin, and α1-acid glycoprotein (Agp)).
Bacillus spp.	**6** Refs: 11028 7822 9079 13145 5416 10752	Supplementation of diets with *Bacillus* can competitively exclude pathogenic bacteria or can be antagonistic toward their growth, thus helping to maintain the health of the intestinal tract. *B. coagulans* couples the production of lactic acid and of two thermostable bacteriocins: coagulin, which showed a bactericidal and bacteriolytic action against different Gram positive bacteria without inhibitory effects on *Lactobacilli,* and a new bacteriocin with antimicrobial activity against Gram (+) and Gram (-) bacteria and fungi. Moreover, the spores of *B. coagulans* could cause immuno-stimulation through their contact with gut-associated lymphoid tissue (GALT), as observed for other *Bacillus* species. *B. subtilis natto* ingestion did not increase IgE antibody levels in the serum, indicating that *B. subtilis natto* was not a food allergen for the calves. *B. subtilis* induces the secretion of serum IgG and Th1 cytokine levels, including IFN-γ, which helps to activate immune systems and enhance immunity. Supplementation of a commercial electrolyte product containing *Bacillus* to scouring calves led to differences in Agp concentration and leukocyte populations. The *Bacillus* promoted development of T-cell subpopulations, including the $\gamma\delta$ T-cell, memory, and activated subsets and regulated inflammation during the scouring event as indicated by the monocyte population (CD8-CD25+, CD8-CD45RO+, CD8-TCR1+). Scour treatment containing electrolyte supplemented with *Bacillus* may provide additional benefits beyond the therapeutic effect, as it may also improve later immune development as evidenced by the enhancement of elements of the innate and adaptive immune systems in calves following the scouring event.
Saccharomyces spp.	**4** Refs: 5416 6684 2914 7582	Supplementation with *Saccharomyces* did not affect animal performance or humoral immune response. Some improvements in neutrophil function (neutrophil chemotaxis and respiratory burst activity) were observed with supplemental yeast culture when cells were incubated with pathogenic *E. coli*. OmniGen-AF® for 60 days prepartum appeared to ameliorate the immunosuppression typically observed during calving. The treated heifers managed the stress associated with parturition better and produced fewer active inflammatory products, hence lower reactive oxygen species (ROS) production. OmniGen-AF® (1) enhanced the function bovine blood leukocytes during the periparturient period, when the innate immune system is typically immunosuppressed and susceptible to new intra-mammary infection (IMI), and (2) tended to reduce the new infection rate at calving.
Pediococcus spp.	**3** Refs: 3302 3301 5416	*Pediococcus* is supplemented in bovine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Clostridium spp.	**3**	*Clostridium* is supplemented in bovine diets as a mixture with other probiotics and prebiotics (synbiotic). Therefore, a specific mode of action could not be attributed to

	Refs: 11692 8737 8736	it.
Propionibacterium spp.	**3** Refs: 4257 2876 3569	Supplementing feedlot cattle diets daily with microbials based on lactic acid- utilising bacteria (*Propionibacterium*) had no effect on ruminal or blood pH, but other variables measured indicated a decreased risk of acidosis. The prevalence of *E. coli* strain O157 in the faeces and on the hide was decreased in steers treated with directfed microbial. This effect on *E. coli* O157 was further supported by a decrease in ileal lamina propria thickness for treated steers, which might indicate that harmful microorganisms capable of causing inflammation were inhibited.
Bifidobacterium spp.	**2** Refs: 9229 491	*Bifidobacterium* is supplemented in bovine diets as mixtures with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Candida spp.	**2** Refs: 5274 9229	Feeding of *Candida* CO119 significantly increased the number of faecal LAB of Holstein calves, suggesting that the bacteria have a probiotic ability to improve intestinal microbial flora. However, the effect was limited in the early stage of the lactation period.
Streptococcus spp.	**1** Refs: 9229	*Streptococcus* is supplemented in bovine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Aspergillus spp.	**1** Refs: 9229	*Aspergillus* is supplemented in bovine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Escherichia spp.	**1** Refs: 5416	*E. coli* is supplemented in bovine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.3.2 Bovine – prebiotics

There are several studies on prebiotic (eight studies; Table 22), such as MOS (two studies) and glucan (two studies). Due to the small number of articles obtained, a summary of the mode of action for each group cannot be described in this report.

Table 22: Mode of action of different prebiotics in bovine

Bovine - Prebiotic	Number of articles	Mode of action
Mannan oligosaccharide (MOS)	**2** Refs: 6033 11129	The highly significant enhancement of IgG level in piglets and calves receiving gut active carbohydrates (GAC) supports the theory that gastric exposure to GAC improves Ig uptake from the gut. Therefore, supplementing neonates with GAC may provide a method for improving immune status and reducing the risk of disease in piglet and calf-rearing operations.
Glucan	**2** Refs: 2860	Glucan is supplemented in bovine diets as a mixture with other prebiotics or other substances and agents. Therefore, a specific mode of action could not be attributed to it.

	11129	
Yeast cell wall (YCW)	**1** Refs: 9229	The addition of prebiotics to the whole milk of dairy female calves increased ADG and reduced faecal shedding of *E. coli,* but dry matter intake and blood parameters related to immune system and cell-mediated immune response were unaffected.
Inulin	**1** Refs: 6937	Inulin feeding could improve effects on iron absorption capabilities. Inulin was able to increase haemoglobin concentration, haematocrit, and IL-10 expression. Therefore, inulin decreased signs of immune activation and increased anti-inflammatory signals.
Cellooligosaccharide (CE)	**1** Refs: 11692	Feed intake, daily gain, and occurrence of diarrhoea in the calves was unaffected by CE supplementation. CE supplementation seemed to have no effect on the maintenance of the levels of *Lactobacillus* and *Bifidobacterium* species in the large intestine of pre-weaning calves.
Lipopolysaccharide (LPS)	**1** Refs: 137	Concentrations of plasma IgG anti-LPS antibodies decreased (P<0.01) and those of IgM anti-LPS antibodies increased (P<0.01) in cows treated with oral LPS. In conclusion, repeated oral administration of LPS from *E. coli* 0111:B4 stimulated a humoral immune response characterised by lower IgG anti-LPS and greater IgM anti-LPS antibodies.

3.4.3.3 Bovine – plant extracts

There area several studies on plant extract (11 studies; Table 23), such as Soybean, *Allium* or *Curcuma longa* derivatives (two studies for each group). Due to the few articles obtained, a summary of the mode of action for each group cannot be described in this report.

Table 23: Mode of action of different plant extracts in bovine

Bovine – Plant extract	Number of articles	Mode of action
Soybean derivatives	**2** Refs: 5866 5911	Feeding fermented soybean meal (FSBM) as calf starter in calves reduced cortisol response and enhanced production of immune-related serum proteins, particularly LPS-specific IgG and IgA, and haptoglobin against LPS challenge. Therefore, FSBM may have beneficial effects on attenuating stress response and enhancing B-cell response partially through the supply of essential amino acids, small peptides, and the enhancement of immune status (humoral response) in weaned calves. Densities of T-lymphocytes were also increased in the jejunal mucosa of calves fed antigenic soya. Increased T-cell densities in the jejunum of calves fed antigenic soya was essentially accounted for by CD8+ cells and WC1+ cells in the epithelium, and CD4+ cells, CD8+ cells and WC1+ cells in the lamina propria. These results suggest the possible implication of a CD8 T-cell-mediated cytotoxicity mechanism at the epithelial level.
Allium derivatives	**2** Refs: 12602 7902	Proportion of CD4+ cells increased in response to garlic. However, induction of the tumour necrosis factor (TNF), IFN-γ, and IL-6 with LPS in vitro was not affected. Therefore, garlic might modulate the function of the adaptive immune system.
Curcuma longa derivatives	**2** Refs: 10046 7902	Proportion of CD4+ cells increased in response to curcumin. However, induction of TNF, IFN-γ, and IL-6 with LPS in vitro was unaffected. Therefore, curcumin might modulate the function of the adaptive immune system.

Cinnamon derivatives	**1** Refs: 12603	A low dose of cinnamon (CIN) tended to increase organic matter (OM) availability in the rumen due to increased feed intake and greater ruminal digested OM. Increased ruminal OM availability did not, however, increase ruminal microbial protein synthesis. In contrast, feed intake and ruminal digestion of feeds was adversely affected when a high dose of CIN was supplemented. The mode of action of CIN appears to be primarily in the rumen, affecting ruminal digestion with minimal effects on intestinal digestion.
Other Chinese herbs	**1** Refs: 8743	The addition of *Fructus Ligustri Lucidi* (FLL) to diet increased apparent total tract digestibility of the diet's dry and organic matter and increased average daily gain and feed efficiency in dairy heifers. Diets supplemented with FLL improved blood antioxidant status (higher concentrations of superoxide dismutase (SOD) and lower concentrations of malondialdehyde (MDA)) and immunity status for dairy heifers (IL-2 increased and IFN-γ, prostaglandin E2 (PGE2) and immunoreactive fibronectin decreased).
Capsicum derivatives	**1** Refs: 7902	The proportion of CD4+ cells increased in response to capsicum. When CD4 T-lymphocytes are activated, they produce cytokines that activate cells of the innate immune system, such as macrophages, and stimulate antibody production from B-cells. However, the induction of TNF, IFN-γ, and IL-6 with LPS in vitro was unaffected. A likely and generally well-accepted mechanism for the pro-inflammatory and pro-oxidative effect of dietary phenolics is hydrogen peroxide generation (i.e. oxygen reduction) coupled with phenolic oxidation. A second potential mechanism that could explain the absence of an observed antioxidant effect of phytonutrients (PN) in this study, and in the case of 8-isoprostane levels a slight increase in pro-oxidant activity, is the generation of o-quinones from phenolic oxidation.
Camellia sinensis	**1** Refs: 10046	Haemoglobin and haemaotcrit values were lower in fermented green tea probiotic (*Camellia*) and the percentage of monocytes and IGM were higher. It is concluded that fermented green tea probiotic may be a suitable alternative to antibiotics for Hanwoo beef calves.
Juniperus communis	**1** Refs: 12602	Supplementation with juniperus improved feed digestibility in the rumen, but possibly at the expense of a reduction in the flow of bypass protein to the small intestine. The total and differential numbers of white blood cells, as well as serum amyloid A and haptoglobin, were unaffected by the treatment, suggesting that the additive had no effect on the immune status of cows.

3.4.3.4 Bovine – animal by-products

There are several of studies on animal by-products (four studies; Table 24), two studies for lactoferrin and two for antibodies. Due to the few articles obtained, a summary of the mode of action for each group cannot be described.

Table 24: Mode of action of different animal by-products in bovine

Bovine – Animal by-product	Number of articles	Mode of action
Lactoferrin	**2** Refs: 10190 8680	Lactoferrin (LF) supplementation did not affect histomorphometrical measures of the intestinal epithelium. However, crypt cell proliferation was negatively affected by Lf supplementation, albeit only in the colon. LF administered orally was shown to act as an immunomodulatory agent by enhancing the size of Peyer's patches in the ileum and

		increasing blood serum immunoglobulin G levels. In addition, the number of peripheral blood leucocytes increased, and mRNA levels of various interleukins such as IL-1β, IL-8, IL-10 and IFN-γ in those cells in response to LF treatment were enhanced. In blood, mRNA expression of the pro-inflammatory marker genes IL-1β and IFN-γ decreased over the 10-week treatment. Additionally, LF feeding decreased villus sizes in the jejunum.
Antibodies	**2** Refs: 5146 3142	IgG absorption by newborn calves is enhanced by adding Se to colostrum. Increased IgG and Se in blood plasma could contribute to improved resistance to infectious diseases in postnatal calves and could reduce the attrition rate.

3.4.3.5 Bovine – other substances

The systematic review reported 20 studies of other substances / agents in bovines. Most of these studies concerned minerals (six studies), lactulose (four studies), vitamins (three studies) and other minor groups (Table 25). Almost all groups of substances /agents include several products, each with different modes of action. Due to the complexity of data and the reduced number of articles in each group, a summary of the mode of action for each group cannot be described in this report.

Table 25: Mode of action of other agents / substances in bovine

Bovine - Other	Number of articles	Mode of action
Minerals	**6** Refs: 5146 5081 10046 12192 3030 1352	**Biotite** (1): enhanced clearance of Bovine herpes virus 1 (BHV-1), a low infection rate of *Mannheimia haemolytica* serotype A1, tempered superficial lesions, and moderated histopathological signs were observed in the germanium biotite supplemented animals after a challenge. **Chromium** (2): Supplementation of the rations for dairy cows with chromium affected the regulation rather than the intensity of immune responses. The relative dominance of the specific antibodies of IgG2 isotype is not only a sign of the prevalence of cell-mediated type of immune response (Th1 type) but also of a higher efficiency in the protection against bacterial and some viral and parasitic infections. For cows fed Cr, concentrations IL-2, IFN-α and TNF-α in the culture supernatants of the mitogen-stimulated mononuclear cells decreased. **Illite** (1): values of haematological indices, differential leukocyte counts, blood proteins, and immunoglobulin among the additive-fed calves were not significantly different. Serum albumin in post-weaning calves of all feed additive groups were similar but significantly lower ($p<0.05$) than in the control group. Post-weaning, IgM was significantly lower ($p<0.05$) in illite-fed calves compared to other treatment groups, but there was no difference pre-weaning. **Selenium** (1): The addition of Se to colostrum might directly activate this physiological pinocytosis of intestinal epithelial cells because of the rapidity of the reaction. Increased IgG and Se in blood plasma could contribute to improved resistance to infectious diseases in postnatal calves and could reduce the attrition rate. **Zinc** (1): Zn source had no influence on the feed intake, milk

		composition, and *S. Pullorum*-challenge in the control group; however, both the Zn-AA W and Zn-Pro S were more effective than Zn-Pro M and Zn sulphate in enhancing rumen fermentation, Zn status, and humoral immune response as well as improving the milk yield of lactating cows (Zn chelates with weak (Zn-AA W), moderate (Zn-Pro M), or strong (Zn-Pro S) chelation strengths). The improved milk production might be attributed to improved rumen fermentation, Zn status, and immune function.
Others	**4** Refs: 8780 6987 12602 118	**Soybean Trypsin Inhibitor** (1): Supplementation of Trypsin Inhibitor in bovine colostrum might improve Ig (IgG and IgM) absorption in calves. Addition of soybean Trypsin Inhibitor may be beneficial not only by protecting Ig but also by protecting these nonspecific antimicrobial factors from proteolytic degradation. **Difructose Anhydride (DFA) III** (1): The MCH was significantly higher in the DFA III group than in the control group. This suggests that DFA III increases Hb production and maintains the MCH within the normal range. Further, supplemental DFA III may accelerate the shift of iron into the Hb in the blood more efficiently in the early stages after calving than in the late stages. Further, the serum iron concentration at 24 h after calving was significantly lower in the DFA III group than in the control group. The increase of IgG indicates the superior immunological status of the calves in the DFA III group at one month after calving, and this finding is consistent with the difference in duration of medical treatment between the groups at two months after calving and at weaning. **Monensin (MO)** (1): Feeding monensin could be beneficial in terms of increasing bypass protein from the rumen, but it did not improve feed digestion or milk production. **Colostrum replacer** (1): Calves fed colostrum replacer had a significantly higher rate of daily weight gain and were less likely to be affected with diarrhoea.
Lactulose	**4** Refs: 3178 3180 3179 6937	The effects of lactulose are sex-specific: male calves tended to have higher body weight and female calves tended to have increased changes in intestinal morphology in response to lactulose. Ileal villus height, crypt depth, and the surface area of lymph follicles from Peyer's patches were reduced by lactulose treatment. Some studies found that lactulose feeding significantly stimulated IL-10 production in the jejunum and colon and others showed decreases in IL-10 mRNA expression. IL-10 can inhibit antigen specific proliferation and cytokine secretion by Th1 lymphocytes and has down- regulatory effects on macrophages and dendritic cells, such as suppression of activation and IL-12 production and prevention of interferon-γ induced disruption of colonic epithelial barriers. High dose lactulose feeding significantly stimulated TGF-β1 production in the caecum. TGF-β1 regulates proliferation, apoptosis, differentiation, and immune regulation. Lactulose has a suppressive effect on the anti-apoptotic marker B-cell lymphoma-extra large (Bcl-xl) in the jejunum. The high lactulose feeding has upregulated the apoptotic molecule caspase 3 in the caecum. The enhanced apoptotic rate of caspase 3 seems to be associated with a decrease in crypt depth due to lactulose supplementation. Total leukocyte count was decreased but a significantly greater number of blood lymphocytes were detected in lactulose group. Additional lactulose feeding had an immunomodulatory effect on the composition of T-cell subsets in different immune compartments. The expression results in male calves indicated that the transcription of IgA Fc receptor in the ileal mucosa of lactulose treatment group increased. Decreases in interferon-γ mRNA expression were observed in the ileum. The CD4-presenting lymphocytes were decreased significantly in the ileum and mesenteric lymph node, whereas CD8-presenting lymphocytes were increased in the blood of females. In some studies, these parameters

		were not modified. Other pro-inflammatory cytokines (IL-1β, IL-8, and TNF-α) and anti-inflammatory cytokines (TGF-β1) did not show significant differences in mRNA expression across treatments. However, IL-8 was significantly upregulated by lactulose in mesenteric lymph nodes in one of the studies. Expression of the lymphocyte activation marker IL-2 receptor alpha chain (IL2RA) tended to decrease in lactulose-treated animals.
Vitamins	**3** Refs: 10190 2860 7582	**Vitamin A** (1): Increased follicle sizes of Peyer's patches (PP) were seen in vitamin A-supplemented calves, and vitamin A selectively affected the number of T-lymphocytes in domes. **Vitamin C** (1): Vitamin C is supplemented in bovine diets as mixtures with other substances. Therefore, a specific mode of action could not be attributed to it.
Organic acids derivatives / Fatty acids	**1** Refs: 6262	**Butyrate**: Exogenous butyrate seemingly had a stimulating effect on the native butyrate-producing bacterial population. This increase was associated with simultaneous reduction in the concentration of both acetate and propionate. Therefore, exogenous butyrate resulted in a significant increase in the relative abundance of Firmicutes, the second most abundant phylum, and a drastic reduction in the most abundant genus, *Prevotella*. However, the relative abundance of the second most abundant genus, *Succiniclasticum*, was relatively unchanged.
Algae	**1** Refs: 4668	Dietary supplementation of sea mustard (*Undaria pinnatifida*) by-product had a significant positive impact on growth performance. β-(1–3)/(1–6) glucans, rich in brown seaweed, are responsible for increased colostral IgG concentrations in seaweed extracts supplemented while IgM concentrations remained unaffected.
Nucleotides	**1** Refs: 6945	The proliferation of peripheral blood mononuclear cells (PBMC) was greater in the uridine monophosphate (UMP) group than in the control group. UMP increased interferon-γ concentration by peripheral blood mononuclear cells (PBMC). Dietary UMP promoted the proliferation of calf T-cells rather than B-cells. UMP may affect the proliferation and differentiation of T-cells either directly or indirectly because pyrimidine-limited conditions alter the balance of Th1/T-helper cell type 2 differentiation. UMP increased the concentration of IgA in the ileum. UMP could affect intraepithelial lymphocytes either directly or indirectly because dietary nucleotides can increase the proportion of the T-cell receptor γδ+ intraepithelial lymphocyte subset and can also upregulate IL-7 production by intestinal epithelial cells.

3.4.4. Ovine and caprine

3.4.4.1 Ovine and caprine – probiotics

There are several studies on probiotics in ovine and caprine populations (16 studies; Table 26), six studies for *Lactobacillus spp.* (four for ovine and two for caprine), and three for *Saccharomyces spp.*. Due to the complexity and the small number of articles obtained, a summary of the mode of action for each group cannot be described in this report.

Table 26: Mode of action of probiotics in ovine and caprine

Caprine & Ovine - Probiotic	Number of articles	Mode of action

Lactobacillus spp.	**6** Refs: 9998 6178 8752 6204 12393 6812	**Ovine** (4): Supplementation with *Lactobacillus* stimulated *Lactobacilli* proliferation, which reached up to 25% of the total bacteria during wheat-induced lactic acidosis. This induced a large increase in lactate concentration, which decreased ruminal pH. Therefore, *Lactobacillus* may be effective in stabilising ruminal pH and therefore preventing sub-acute ruminal acidosis (SARA) risk (reduction of the occurrence of butyric and propionic), but they were not effective against lactic acidosis. *L. acidophilus* also enhanced immune-regulatory functions. Lactic acid bacteria had a protective effect against Transmissible Gastroenteritis Coronavirus (TGEV) and Rotavirus (RV) on animal and human intestinal and macrophage cell lines of non-tumour origin and it reduced the total number of *E. coli* O157:H7 shed in the faeces and improved animal meat production in lambs previously infected. **Caprine** (2):*L. plantarum* led to increased LAB and reduced clostridia faecal counts, underlining a possible beneficial probiotic effect in balancing the goat intestinal microbiota toward potentially beneficial microbes. The reduction in clostridia could also imply a protective effect against clostridial intestinal disorders and diarrhoeas. The antioxidant capacity and the concentrations of immunoglobulins IgA, IgM and IgG in goat plasma did not differ across treatments. The strain showed an interesting potential toward the enrichment of milk in beneficial polyunsaturated fatty acids.
Saccharomyces spp.	**3** Refs: 6736 8752 12210	Inter Yeast S. stimulates specific and non-specific humoral and cellular immunity in lambs (it stimulated the proliferative activity of T- and B-cells). With regard to humoral immunity parameters, significantly higher gamma globulin levels and higher lysozyme and ceruloplasmin activity were found in the blood serum of experimental lambs that were administered the Inter Yeast S. An analysis of cellular immunity indicators revealed significantly higher levels of respiratory burst activity (RBA) and potential killing activity (PKA) of phagocytes, as well as higher proliferative response of blood lymphocytes after stimulation with LPS and ConA.
Enterococcus spp.	**2** Refs: 6811 12393	Pre-treatment with *E. faecium* PCK38, prior to viral challenge, translated into a marked increased in survival percentages as well as a protective effect against monolayer disruption (40% survival, i.e. four times higher than the survival obtained with the virus application).
Bacillus spp.	**1** Refs: 5741	Milk yield, fat, and protein content were significantly increased after the addition of probiotics (*Bacillus* strains).
Bifidobacterium spp.	**1** Refs: 9998	Lambs fed milk replacer (MR) containing a mix of *Bifidobacteria* showed the highest in vivo cellular immune response to phytohemagglutinin. Finally, it is concluded that *B. animalis* subspp. *lactis* and *B. longum* subspp. *longum* improved humoral response in lambs.
Streptococcus spp.	**1** Refs: 6178	Supplementing the diet of lambs infected with *E. coli* O157:H7 with *S. faecium* can reduce the total number of *E. coli* O157:H7 shed in the faeces and can improve animal meat production.
Aspergillus spp.	**1** Refs: 12393	*Aspergillus* is supplemented in caprine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Propionibacterium spp.	**1** Refs: 6204	*Propionibacterium* probiotic strains may be effective in stabilising ruminal pH and therefore preventing SARA risk, but they were not effective against lactic acidosis.The effectiveness of probiotics is compromised by ruminal fermentations, and they are effective when the ruminal ecosystem is unstable. Probiotics stimulated *Lactobacilli* proliferation, which reached up to 25% of total bacteria during wheat-induced lactic acidosis. This induced a significant increase in lactate concentration, which decreased ruminal pH.

3.4.4.2 Ovine and caprine – prebiotics

There are several studies on prebiotics in ovine and caprine populations (seven studies; Table 27), and two studies for MOS. Due to the small number of articles obtained, a summary of the mode of action for each group cannot be described in this report.

Table 27: Mode of action of prebiotics in ovine and caprine

Caprine & Ovine – Prebiotic	Number of articles	Mode of action
Mannan oligosaccharide (MOS)	**2** Refs: 12474 5507	The presence of MOS prebiotic in ewe diets can influence some blood indices. When introduced to the rations based on meadow hay and grass-alfalfa haylage, this prebiotic elevated haematocrit value and red and white blood cell count, with slightly decreased total cholesterol and triglyceride levels in blood plasma. Supplementation of this prebiotic to the diets based only on hay (without haylage) did not significantly change these parameters. Irrespective of the feeding model, the additive increased the level of alkaline phosphatase by about 31%.
Glucan	**1** Refs: 12474	Glucan is supplemented in ovine diets as a mixture with other prebiotics. Therefore, a specific mode of action could not be attributed to it.
Fructooligosaccharide (FOS)	**1** Refs: 12393	FOS is supplemented in caprine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Yeast cell wall (YCW)	**1** Refs: 12474	YCW is supplemented in ovine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Inulin	**1** Refs: 5206	Inulin had no significant effects on selected faecal bacterial populations, BW, haematological parameters, health status orthe incidence of diarrhoea. A dailydose (0.6 g) of inulin might not be sufficient to yield observable immunological effects.
Inactivated yeast-bacteria	**1** Refs: 7239	Administration of *Saccharomyces cerevisiae* dried brewer's yeast to suckling lambs has a stimulating effect on their meat performance traits, lysozyme, ceruloplasmin activity, and increased serum concentrations of gamma globulins.

3.4.4.3 Ovine and caprine – plant extract

There is only one study about plant extracts (*Astragalus* derivatives) in ovine and caprine populations (Table 28). Due to the small number of articles obtained, a summary of the mode of action for each group cannot be described in this report.

Table 28: Mode of action of plant extracts in ovine and caprine

Caprine & Ovine – Plant extract	Number of articles	Mode of action
Astragalus derivatives	**1** Refs: 12959	*Astragalus* polysaccharide (APS) feeding did not affect the immune responses of lambs; a possible reason for this may be that the supplemented dose was suboptimal. However, *Astragalus* membranaceus root (ATM) dose did affect the immune responses of lambs, including inducing IgM productions and lymphocyte proliferation, and these responses may protect lambs against pathogenic and non-pathogenic immune challenges because the main antibody produced in primary immune responses to antigens is IgM. APS and AMT exhibited consistent antioxidant effects in newly weaned lambs by increasing T-SOD activities in blood and subsequently increasing their total antioxidant status. Reduced blood cortisol concentrations of lambs fed APS and ATM support affects APS and ATM with regard to regulation of antioxidant status.

3.4.4.4 Ovine and caprine – animal by-products

There is only one study on animal by-products (lactoferrin) in ovines and caprines (Table 29). Due to the small number of articles obtained, a summary of the mode of action for each group cannot be described in this report.

Table 29: Mode of action of animal by-products in ovine and caprine

Caprine & Ovine – Animal by-product	Number of articles	Mode of action
Lactoferrin	**1** Refs: 12563	Lactoferrin has a direct antimicrobial effect on *E. coli* O157:H7 and is responsible for the proteolytic degradation of EspA and EspB (two structural proteins of the bacterial type III secretion system). In addition, lactoferrin is an immunomodulatory protein. The results suggest that oral lactoferrin administration could be used to prevent persistent colonisation of sheep with *E. coli* O157:H7.

3.4.4.5 Ovine and caprine – other substances

The systematic review reported five studies of other substances / agents in ovine and caprine populations. Most of these studies concerned minerals (two studies) and other minor groups (Table 30). Due to the reduced number of articles in each group, a summary of the mode of action for each group cannot be described.

Table 30: Mode of action of other substances / agents in ovine and caprine

Caprine & Ovine - Other	Number of articles	Mode of action

Minerals	**2** Refs: 8752 12159	**Selenium**: Comparing different types of Se supplementation, Se-enriched probiotics seemed to be more effective than sodium selenite in increasing blood GSH-Px activities in lambs. Supplementation of Se in the lambs' diet led to improvement of IL-1 and IL-2 levels in plasma. However, there seem to be no significant differences for IL-1 and IL-2 levels in plasma of lambs supplemented with an organic or inorganic Se source. The interleukins-increased lambs remained in good health during the entire study, showing that the increase of interleukins was a moderate increase and indicating that the immune function of the lambs was enhanced upon Se supplementation. In neonatal pigs, increased inflammatory signalling was detected in the HSe-HIH group (high selenium and 140% of the nutritional requirements), as indicated by increased mRNA expression of TNF-α and chemotaxis IL-8. Consistently, phosphorylation of c-Jun N-terminal kinase, a primary inflammatory signalling mediator, was greater in the High Se-HIH group compared with other treatments. Consistent with cytokine expression, mast cell density was less in the High Se-CON (normal requirements) group than in other treatments. The expression of TNF-β mRNA was greater in the HSe-HIH group; consistently, collagen content was increased in the HSe-HIH group compared with the Adequate Se-CON group. In conclusion, independently, neither High Se nor HIH had major effects on inflammation but, in combination, these maternal treatments induced an inflammatory response in the neonatal intestine.
Vitamins	**1** Refs: 7401	**Vitamin E**: Vitamin E supplementation tended to reduce the increments of blood tissue damage indicators during road transport (creatine phosphokinase), thus preserving the health of the animal under stress conditions.
Enzymes	**1** Refs: 12393	Amylase is supplemented in ovine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Other	**1** Refs: 7401	**Carnosic acid**: Carnosic acid promoted changes in the faecal bacterial community, which might be related to differences in feed digestion in the large intestines.

3.4.5. Fish

3.4.5.1 Fish – probiotics

3.4.5.1.1 Salmonids

Table 31: Mode of action of different probiotics in fish (Salmonids)

Salmonids - Probiotic	Number of articles		Mode of action
	FISH	SALMONIDS	
Lactobacillus spp.	54	**15** Refs: 617 8184 612 11579 7767 8186 8183	A higher percentage of cells were phagocytic in the probiotic fed group. *Lactobacillus* had an effect on the fish complement bactericidal activity, confirming the benefit for the non-specific humoral defence system. This antibacterial effect was also observed by the higher IL-1β1 expression in both the spleen and kidney. Lactobacilli compete with the pathogen in adherence to the mucus layer by producing bacteriocins which would inhibit pathogenic colonisation. Supplementation of LAB in the diet induced greater levels of immunoglobulin and lysozyme activity. The haemaotcrit values were

		8185 11884 10242 8187 8447 616 613 9470	also increased, implying improved health status. Up-regulation of TGF-β gene, which is an important cytokine, was responsible for several cellular immune functions.
Lactococcus spp.	11	**7** Refs: 8447 610 616 617 612 613 11884	Probiotic bacteria can create a hostile environment for the pathogen by interfering with pathogen colonisation and can also modulate the immune response (enhancement of phagocytic activity of the head kidney leukocytes).
Bacillus spp.	58	**6** Refs: 3666 1270 8185 7176 7178 7707	*Bacillus spp.* levels dominated both the mucosal and digesta microbial populations (>70%). Dietary application of BioPus 2B can result in high numbers of *B. subtilis* and *B. licheniformis* surviving passage through the upper gastrointestinal tract to the posterior intestine, where the potential for colonisation is demonstrated by the high levels associated with the mucosa. The higher number of mucin-producing goblet cells in rainbow trout fed a diet supplemented with the probiotic might be due to the observed change in the gut microbiota as goblet cells are modulated by the presence and abundance of microbial organisms. The observed increase in goblet cells, and the larger infiltration of leukocytes in the lamina propria of villi from fish fed the diet supplemented with probiotic, would support the plausibility of an enhanced immune response in fish. The mode of action reflected nutrition, production of inhibitory substances, and stimulation of the innate immune response. Specifically, JB-1 and GC2 were positive for siderophore and chitinase production as well as increased lysozyme, phagocytic, and respiratory burst activities. *B. subtilis* AB1 stimulated both cellular and humoral immune responses, which may provided protection to survive infection by the highly virulent *Aeromonas spp.*
Enterococcus spp.	9	**5** Refs: 7176 7178 7117 8185 10242	*E. faecium* levels dominated both the mucosal and digesta microbial populations (>70%). The high intestinal levels, in terms of the relative percentage of intestinal microbiota, are likely due to several factors: (i) E. faecium grows well at rainbow trout-rearing temperatures (as low as 12 ºC); (ii) *E. faecium* has been shown to be antagonistic to genera that are often indigenous to the gut (e.g. *Aeromonas spp.*); and (iii) *E. faecium* can tolerate exposure and has good adhesive properties to intestinal mucous. *E. faecium* induced significantly higher IL-1β1 expression in both the spleen and kidney, implying a probiotic involvement through this mediator of immune response. The IL-1β1 molecule is known to exert both direct and indirect antibacterial effects in vivo in mammals.
Leuconostoc spp.	5	**5** Refs: 8447 610 616	Not clear. The additive is used as part of aprobiotic mixture. Therefore, the mode of action of the probiotic on its own could not be evaluated.

		617 613	
Aeromonas spp.	7	**4** Refs: 1324 1270 4755 1268	*Aeromonas* bacterial isolates were identified as probiotic candidates with the potential to control or reduce disease caused by *F. psychrophilum. A. sobria GC2* was also beneficial to rainbow trout when administered as a feed supplement for the control of lactococcosis and streptococcosis. Aeromonas was positive for siderophore and chitinase production as well as increased lysozyme, phagocytic and respiratory burst activities.
Carnobacterium spp.	5	**4** Refs: 4755 9098 5376 5375	*Carnobacterium* (BA211) reduced leucocytes and increased in the number of erythrocytes, kidney macrophages, phagocytic and respiratory burst activity of head kidney macrophages, lysozyme activity of serum and gut mucus, and the proportion of lymphocytes to monocytes. It also induced the mRNA expression level of pro-inflammatory cytokines, IL-1β and TNF-α, from rainbow trout Head Kidney (HK) leucocytes. Carnobacterial cultures were beneficial for rainbow trout in terms of resisting challenge to *A. salmonicida* and *Y. ruckeri* and enhancing cellular and humoral immune responses. Co-incubation of the foregut with a pathogen and *C. divergens* did not reverse the damaging effects caused by the pathogen, although these were alleviated when probiotic bacteria were used. To some extent, this might be attributable to the antagonistic activity of the probiotic bacteria against the pathogens, resulting in fewer live pathogens available to colonise the foregut and therefore less tissue damage.
Micrococcus sp	4	**3** Refs: 10390 10391 10392	Live cells of *Kocuria* SM1 protected rainbow trout against infection from *V. anguillarum* and *V. Ordalii* (reduction in mortalities). SM1 stimulated both cellular and humoral immune responses in rainbow trout by elevation of leucocytes, erythrocytes, protein, globulin and albumin levels, upregulation of respiratory burst, complement, lysozyme, peroxidase, and bacterial killing activities.
Pediococcus spp.	10	**2** Refs: 7177 13095	*P. acidilactici* temporarily colonised the digestive tract during supplemented feeding. Increased leucocyte levels were observed in fish fed *P. acidilactici* supplemented diet, yet leucocyte types were unaffected.
Saccharomyces spp.	16	**2** Refs: 11580 10242	Diets supplemented with 2ME-treated yeast stimulate the immune system (increasing neutrophil count, enhancing serum lysozyme and complement activity, increase in alternative complement pathway activity) and growth of juvenile rainbow trout, thus enhancing their resistance to *Y. ruckeri*. Furthermore, treating *S. cerevisiae* with 2ME improved digestibility, leading to improved utilisation of yeast cell nutrients. HUFA-enrichment of this yeast was not efficient enough for augmentation of immunity in rainbow trout.
Vibrio spp.	6	**1** Refs: 4755	Leucocytes counts were reduced when *Vibrio* was added to the diet. There was evidence for an increase in the number of erythrocytes and kidney macrophages as well as the proportion of lymphocytes to monocytes.
Pseudomonas spp.	5	**1** Refs: 5684	*Pseudomonas* M162 had no unfavourable effects on the health of the fish, survived in the gastrointestinal track of the fish, and inhibited the growth of *Flavobacterium psychrophilum* in vitro and improved resistance to it in vivo. One of the modes of action of this probiotic was its immunostimulatory effect. M162 had significantly higher levels of leucocytes and serum lysozyme activity and also enhanced total IgM production in rainbow trout.

Methylococcus spp.	1	**1** Refs: 9219	Supplementing diets with a bacterial meal containing mainly *Methylococcus capsulatus* seemed to have a protective effect against soybean meal-induced enteritis in salmon. The level of inflammatory regulators (CD8a+ T-lymphocytes at the base of the epithelial cells and MHC-II reactive cells in the lamina propria and submucosa) was normalised by adequate dietary inclusion of bacterial meal.
Luteimonas spp.	1	**1** Refs: 1064	*Luteimonas* is supplemented in fish (salmonid) diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Rhodococcus spp.	1	**1** Refs: 1064	*Rhodococcus* is supplemented in fish (salmonid) diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Microbacterium spp.	1	**1** Refs: 1064	*Microbacterium* is supplemented in fish (salmonid) diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Sphingopyxis spp.	1	**1** Refs: 1064	*Sphingopyxis* is supplemented in fish (salmonid) diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Leucobacter spp.	1	**1** Refs: 1064	*Leucobacter* is supplemented in fish (salmonid) diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Dietzia spp.	1	**1** Refs: 1064	*Dietzia* is supplemented in fish (salmonid) diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Plesiomonas spp.	1	**1** Refs: 1324	*Plesiomonas shigelloides* was identified as a probiotic candidate with the potential to control or reduce disease caused by *F. Psychrophilum.*
Hafnia spp.	1	**1** Refs: 1324	*Hafnia alvei* was identified as a probiotic candidate with the potential to control or reduce disease caused by *F. Psychrophilum.*
Enterobacter spp.	1	**1** Refs: 1324	*Enterobacter spp.* was identified as a probiotic candidate with the potential to control or reduce disease caused by *F. Psychrophilum.*
Citrobacter spp.	1	**1** Refs: 1324	*C. freundii* was identified as a probiotic candidate with the potential to control or reduce disease caused by *F. Psychrophilum.*
Lysinibacillus spp.	1	**1** Refs: 1324	*L. fusiformis* was identified as a probiotic candidate with the potential to control or reduce disease caused by *F. Psychrophilum.*
Staphylococcus spp.	1	**1** Refs: 1324	*S. equorum* was identified as a probiotic candidate with the potential to control or reduce disease caused by *F. Psychrophilum.*
Pantoea spp.	1	**1** Refs: 10630	The inclusion of LPS derived from the cell walls of *P. agglomerans* (IP-PA1) in diets for trout fingerlings promoted growth, improved feed utilisation, enhanced non-specific immune response, and promoted gut immunity by increasing goblet cell number. It increased bactericidal activity in serum, lysozyme activity, and

			haemolytic complement activity values.

3.4.5.1.2 Freshwater fish

Table 32: Mode of action across different probiotics in fish (Freshwater fish)

Fresh water fish - Probiotics	Number of articles		Mode of action
	Fish	Fresh water fish	
Bacillus spp.	58	**16** Refs: 11306 9024 587 12545 12299 6346 1666 2298 3533 7666 5811 12877 117 9080 116 4219	*Bacillus* as a probiotic food additive could modulate intestinal microflora, enhance digestive enzyme activities and improve growth parameters of fish. Dietary *B. subtilis* C-3102 induced upregulation of intestinal cytokine expression (IL-1β, TGF-β and TNF-α) and downregulation of intestinal Heat shock protein (HSP)-70. However, no observable colonisation of *B. circulans* was found in the hindgut of fish. The inclusion of the probiotic bacteria *Bacillus* benefited the innate immune system of fish by increasing the mean corpuscular haemoglobin, and improving the innate immune system (increase in WBC, serum protein, globulin content lysozyme, phagocytic activities of macrophages, complement, respiratory burst and bactericidal activity, SOD, GPX, Catalase (CAT) and immunoglobulins). Total protein, globulin and IgM of fish fed *B. licheniformis* supplemented diets were also increased, indicating the enhanced immune function of triangular bream. Fish fed *B. thuringiensis, B. coagulans and B. amyloliquefaciens* had higher survival rates, which indicated enhanced immunity and disease resistance against *Aeromonas hydrophila, A. Veronii* infection, or bacterial challenge, respectively. This can be explained on the basis of increased bactericidal activity of serum. However, *B. toyoi* did not protect the test eels from *E. tarda*.
Lactobacillus sp	54	**13** Refs: 4141 9469 1374 3662 4896 117 7729 8564 9016 6421 8563 116 3636	Supplementation with *Lactobacillus* fish diets can improve growth and innate immune response (increases respiratory burst, phagocytic, alternative complement pathway, lysozyme activity and IgM). *Lactobacillus* also improved the gut bacterial microbiota (viable culturable heterotrophic bacteria counts were reduced, and the number of lactic acid bacteria was increased) improving the intestinal structure (increasing the villus height) and its mucosal immunity (higher level of intraepithelial lymphocytes and the higher level of acidophilic granulocytes). It also increased the number of circulating thrombocytes, total leukocytes, and lymphocytes and it induces IL-1 and TNF-α cytokines in fish. *Lactobacillus* may reduce the oxidative stress and, in turn, the hepatic cell death confirming enhancement of the intestinal innate immune response. *Lactobacillus* also provides protection against pathogens and promotes survival. Both improved host immunity and competition for adhesion sites with the pathogen contributing to the beneficial effects of *Lactobacillus*.
Saccharomyces spp.	16	**6** Refs:	Yeast can enhance performance, feed utilisation, immunological responses, and can improve their challenge against infection.

		6255 1577 117 13085 4218 6256	DVAQUA (*Saccharomyces cerevisiae* fermentation product) increased intestinal bacterial count and bacterial diversity. Complement component concentrations and head kidney macrophage phagocytic index were also increased. Fish fed diets supplemented with brewer's yeast had higher serum peroxidase levels.
Vibrio spp.	6	**3** Refs: 1816 4756 2583	*A. veronii* BA-1 was beneficial for carp during resisting challenge to a pathogen and enhancing cellular and humoral immune response. Respiratory burst activity was unaffected, but cytotoxic activity had increased.
Pediococcus spp.	10	**2** Refs: 3116 10862	Successful temporal colonisation (i.e. with continual dietary supplementation and for several weeks after administration) of the GI tract by *P. acidilactici* appeared to cause a general trend toward elevated growth, survival, and immunostimulation (modulates both localised intestinal and peripheral innate immunity). *P. acidilactici* did not affect tilapia gross morphology or intestinal immune cells. *Pediococcus* increased in the total number of circulating leucocytes and reduced erythrocyte size and number, which resulted in a lower haematocrit value. A significant increase in serum lysozyme activity was also observed in the fish fed the *P. acidilactici* supplemented diet. TNF-α production was also increased, which initiated a cascade of cytokines that subsequently recruit macrophages and neutrophils to the site of inflammation.
Aeromonas spp.	7	**2** Refs: 1816 4756	*A. veronii* BA-1 was beneficial for carp during resisting challenge to *A. hydrophila* and enhancing cellular and humoral immune responses. However, cellular rather than humoral immunity is a factor in explaining the benefit of these inactivated bacterial cell preparations.
Pseudomonas spp.	5	**2** Refs: 13076 3661	For fish fed diets supplemented with *Pseudomonas spp.,* it seems that this species was not suitable as a probiotic for Nile tilapia. However, it was observed that dietary supplementation of *P. aeruginosa* VSG-2 could improve the innate immunity (increased serum lysozyme and alternative complement pathway (ACP) activities, phagocytosis, and respiratory burst activity in head kidney macrophages and superoxide dismutase (SOD) activity) as well as survival of *L. rohita* against *A. hydrophila* infection.
Enterococcus spp.	9	**1** Refs: 1666	*E. faecium* SF68 protects the test eels from *E. tarda* challenge. The survival rates of eels fed with *E. faecium* SF68 supplement was significantly higher. *E. faecium* SF68 antagonises rather than inhibits *E. tarda*. Ensuring these two probiotics are the dominant microflora in the intestine prior to pathogen invasion may be the key to disease prevention.
Lactococcus spp.	12	**1** Refs:	*L. lactis spp. lactis* ST G45 is capable of surviving and colonising the fish intestinal mucus as well as antagonising the resident microbiota.
Carnobacterium spp.	5	**1** Refs: 4756	Cellular rather than humoral immunity is a factor in explaining the benefit of inactivated *Carnobacterium* BA211 cell preparations.
Micrococcus spp.	4	**1** Refs: 13076	*M. luteus* isolate is beneficial for Nile tilapia when administered as a feed-additive, enhancing the growth performance and the fish's resistance against *A. hydrophila* infection.
Aspergillus spp.	2	**1**	*Aspergillus* is supplemented in fish (freshwater fish) diets as a mixturewith other probiotics. Therefore, a specific mode of

		Refs: 117	action could not be attributed to it.
Weixella spp.	1	**1** Refs: 7466	The haematological and immunological (increased total immunoglobulin concentration in the blood serum) parameters of *Pseudoplatystoma* hybrids showed a positive response to the supplementation of *W. cibaria* when supplemented for 15 days via diet.
Flavobacterium spp.	1	**1** Refs: 1816	*F. sasangense* BA-3 was beneficial for carp during resisting challenge to *A. hydrophila* and enhancing cellular and humoral immune response.
Rhodopseudomonas spp.	1	**1** Refs: 12978	Treatment with *R. palustris* G06, as water additives, could be used to enhance immune (increase immune responses such as myeloperoxidase (MPO) activity, respiratory burst activity, SOD activity and CAT) as well as health status, thereby improving growth performance of *O. niloticus*.

3.4.5.1.3 Marine fish / shellfish

Table 33: Mode of action of different probiotics in fish (Marine fish / Shellfish)

Marine fish /shellfish - Probiotic	Number of articles		Mode of action
	Fish	Marin fish /shellfish	
Bacillus spp.	58	**36** Refs: 12906 2280 7662 12581 12216 6248 129 11042 12622 9025 2281 1605 1606 1607 1609 12905 12908 13021 13023 13185 6387 12953 614 9466 9467 4145 4146 7772	Improvements in growth parameters and survival of **shrimp larvae** were observed when *Artemia* was enriched with *Bacillus* or *Bacillus* was added to rearing water. Significant improvements in microvilli height and density were also observed when *Artemia* was enriched with *Bacillus*. Adding the probiotic to the shrimp larvae rearing water produced weak inhibition of bacterial growth. Gene expression of prophenoloxidase I, prophenoloxidase II, and lysozyme of larvae were significantly increased after being reared in probiotic-containing water. *Bacillus* boosted growth as well as the immune responses of the **shrimp** (increasing phenoloxidase activity, lysozyme activity, nitric oxide synthase activity, superoxide dismutase activity, total haemocyte count, respiratory burst activity, plasma protein concentration, and bactericidal activity). Secretion of digestive enzymes in the GI tract could be another possible explanation for the increased growth performance, which consequently results in optimal health and higher survival rates. *Bacillus* also modulates intestinal microflora and can significantly improve disease resistance (reduced mortality and colonisation after a challenge) and can protect shrimp from pathogenic bacterial infection by competitive exclusion. **Marron** fed *Bacillus* supplemented diets produced beneficial outcomes in terms of physiological condition (organosomatic indices), immune parameters (total haemocyte count (THC), different haemocyte count (DHC), granular cells proportion, and bacteria load in haemolymph); the level of the bacteria load in the

		4123 2957 9468 615 1610 5809 6404 11549	intestine is also affected. Probiotics to larval ***H. gammarus*** reared with microalgae improved growth, survival, and microbial parameters. The individual supplementation of *Bacillus* indiets produced increased resistance to stress, gastrointestinal and bacterial communities, and survival. Supplementation with *Bacillus* improved growth and could increase disease resistance in **fish** through the stimulation of both the cellular and humoral immune function, such as phagocytic, lysozyme, complement, superoxide dismutase, respiratory burst, peroxidise activities and IgM. Supplementation with *Bacillus* also increased the expression of IL-8, Caspase (CASP)-1, Cyclooxygenase (COX)-2 and transferrin. *Bacillus* also shapes the intestinal microbiota and mucosal immune gene expression in fish (especially the significantly upregulated expression of antibacterial peptides, which may play an important role in the intestinal microbiota suppressing pathogenical bacteria). In sea bream, supplementation with *Bacillus* reduced microbiota diversity and decreased microvillus. *B. subtilis* can improve disease resistance by enhancing immunity, as well as presumably modulating microflora in the **sea cucumber's** gut. *B. subtilis* increased the specific growth rate (SGR), total coelomocytes counts (TCC), phagocytosis of sea cucumbers, the counts of total viable bacteria, and disease resistance to *V. splendidus*, whereas the *Vibri counts* decreased.
Lactobacillus spp.	54	**26** Refs: 6107 8514 13093 4139 2315 9051 9468 10746 868 870 869 3317 1844 5643 9048 9049 4144 4138 4145 4146 8515 956 8404 9466 9467 2229	The early colonisation (during gut metamorphosis) of the intestine by exogenously administered autochthonous bacteria *Lactobacillus* stimulates expansion of T-cells and acidophilic granulocytes (AGs) in the mucosa without affecting the integrity of the gut and lowered transcription of key pro-inflammatory genes. The decreased TGF-β and IL-10 transcripts (correlated to lower IL-1β expression) could play a role in the increase of T-cells. Feeding with *Lactobacillus*-supplemented diet also increased the number of Ig+ cells and acidophilic granulocytes in the gut, and the effects were more pronounced when administration began during gut metamorphosis. *Lactobacillus* enhances the growth performance and the immune system of **fish**. The viable *Lactobacilli* in the posterior intestines of fish fed the *L. plantarum*-containing diets had dominantly increased. The digestive ability of fish appeared to be improved by microbial preparations through an increase in trypsin and acid phosphatase synthesis as well as a modification of the endocytosis apparatus. *Lactobacillus* strains may act as immune-modulators because they enhance IgM, catalase (CAT) and superoxide dismutase (SOD) in fish and are important for pathogen recognition and activation of the innate immune system via the classical pathway of complement activation. Phagocytic, respiratory burst, alternative complement activity (ACH50), cytotoxic and lysozyme activities were increased in fish fed diets supplemented with *Lactobacillus*. Up-regulation of pro-inflammatory (IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N), cell-mediated immunity inducing (IL-12p35, IL-12p40 and IL-18), antiviral/intra-cellular pathogen killing (I-IFN-

			1 and IFN-g), anti-inflammatory (IL-10) and peripheral T-cell expansion and survival controlling (IL-2, IL-7, IL-15, IL-21 and TGF-β1) cytokines were observed in the treated fish. Fish feeding *Lactobacillus* enriched diet affords higher levels of disease protection (reduced mortality) due to stimulation of the immune system. Resistance against bacterial and viral pathogens is correlated with increases in ACH50 and lysozyme activity of fish fed *Lactobacillus* supplemented diets. *Lactobacillus* could be used to improve the host-associated microflora (increase LAB population), growth, feed efficiency, carcass biochemical composition, and immune response of giant freshwater **prawn**. However, *Lactobacillus* failed to maintain its population in the digestive tract of the animal when the prawns were fed anunsupplemented diet, indicating the temporary colonisation ability of the probiotic bacteria. The immune parameters total hemocyte count (THC), phenol oxidase (PO) activity, respiratory burst (RB) activity, SOD activity, prophenoloxidase (proPO), Peroxinectin (PE) mRNA transcription, and clearance efficiency increased with concurrent decrease in cumulative mortality against a challenge.
Saccharomyces spp.	16	**8** Refs: 1845 4138 4145 4146 10183 8040 8404 2495	*S. cerevisiae* administration to **fish** in order to promote growth induces upregulation of innate cellular and humoral immune responses and resistance to a pathogen. The phagocytic activity, respiratory burst, and superoxide dismutase (SOD) level of head kidney leucocytes, as well as serum lysozyme activity, serum alternative complement activity (ACH50), and cytotoxic activity of fish fed diets containing *S. cerevisiae* were increased. The DVA (yeast) extended positive effects on growth, improved sediment quality, reduced endotoxin in **shrimp** intestine, and enhanced activities of lysozyme and PO. In shrimp, *S. cerevisiae* also yielded increased survival after a challenge, and there does appear to be an immunostimulatory effect.
Pediococcus spp.	10	**6** Refs: 7680 4571 12699 1569 8406 2229	*P. acidilactici* improved the growth rate and the innate immune system (Ig and lysozyme). *Pediococcus* enhanced the growth rate of groupers and shrimp as well as the erythrocyte counts; it also elevated the proliferation of leukocyte, respiratory burst (RB) of peripheral blood leukocytes, and phagocytic activity of head-kidney phagocytes post- infection, as well as protecting the groupers from vibriosis.
Shewanella spp.	6	**6** Refs: 129 4123 4960 4011 2957 9468	Dietary supplementation of *Shewanella* in **marron** had no significant impact on growth but was effective in improving the resistance of marron to *V. mimicus*. *S. haliotis* can promote growth and resist challenge to *V. harveyi* in *L. Vannamei*. It enhanced both respiratory burst and superoxide dismutase activities. **Abalone** fed diets containing *S. colwelliana* WA64 and S. *olleyana* WA65 had led to an enhanced cellular and humoral immune response, notably higher haemocytes, respiratory burst activity, serum lysozyme activity, and total protein levels. Mortality after the challenges with *V. harveyi* was reduced in abalone fed diets supplemented

			with the probiotic. Dietary administration of *Shewanella putrefaciens* to **gilthead sea bream** promoted growth and stimulated some of the immune activities (decreased serum IgM levels and peroxidase activity). *Shewanella* putrefaciens may serve as good natural antioxidants for farmed fish, as a preventive measure for protection against free radical-induced disorders. *Shewanella* 51M6 appear to possess good immune stimulatory properties in vitro, while *Shewanella* Pdp11 produced weaker effects on the sea bream cellular innate immune parameters.
Lactococcus spp.	12	**4** Refs: 5374 11039 6678 4297	Dietary administration of *L. lactis* and *E. faecium* significantly improved the feed efficiency of fish while slightly decreasing the growth and feed intake. Disease protection is due to the flounder's innate immunity, activated by the *L. lactis* administration: increased lysosomal activities and production of IL-12 and IFN-γ. L. *lactis* has a strong antibacterial activity against *Streptococcus iniae*, *Streptococcus parauberis* and *Enterococcus viikkiensis*, and moderate activity against *Lactococcus garviae*. *Lactococcus* also modulates the immune function in fish and shrimp (increased the serum lysozyme activity and complement C3 level, antiprotease, serum peroxidase and blood respiratory burst activities).
Enterococcus spp.	9	**3** Refs: 6012 2229 11039	The *Enterococcus* strain was effective in maintaining lower *Vibrio* levels in the gut, enhancing juvenile growth, and leading to increased survival. *Enterococcus* strain had a broader inhibitory potential, inhibiting *Aliivibrio* (*Vibrio*) *salmonicida*, *Aeromonas salmonicida* subspp. *achromogenes* and *V.anguillarum*. *E. faecium.* MM4 also modulates the immune function in *E. coioides* (affecting the serum lysozyme activity and complement C3 level).
Debaryomyces spp.	3	**3** Refs: 10048 9052 9050	Application of *Debaryomyces hansenii* (S8) helps enhance disease resistance through stimulation of the nonspecific immune system of the **prawns** (increase in alkaline phosphatase activity). Cellular innate immune parameters were positively modulated in **fish** by the *D. hansenii*-supplemented diet. Enhanced ROS production and peroxidase activity in HK leucocytes during oxidative burst were increased, which suggests that an enhanced leucocyte microbe-killing capacity is a key factor in increasing resistance to disease. *D. hansenii* L2 also indicated a stimulatory effect in the phagocytic capacity of HK phagocytes in their phagocytic ability. Yeast-supplemented diets up-regulated the expression of 12 genes (IgM, MHC-Ia, MHC-IIa, C3, IL-1β, TLR, TNF-α, Colony stimulating factor (CSF)-1R, Non-specific cytotoxic cell receptor (NCCRP)-1, Hep, TCR-β and CD8 genes) after two weeks, especially in the skin, intestine, and the HK. Liver, only C3 gene expression was up-regulated by the experimental diet. The level of C3 mRNA showed a much higher increase in the intestine than in HK and liver. The addition of *Debaryomyces* to the feed could also exert an important influence on intestinal bacterial groups and could yield faster homogenisation of the intestinal microbial community.
Psychrobacter spp.	3	**3** Refs:	The enhanced specific activities of digestive enzymes may be induced by the probiont and may contribute to improved feed utilisation. Dietary administration of

		6028 11041 11036	*Psychrobacter spp*. enhanced feed utilisation and immune responses (slightly enhanced serum SOD activity, a slight increase in phagocytic activity, phagocytic index, and serum complement C3 and C4 also increased, which suggested that *Psychrobacter spp.* may activate the complement system) of *E. coioides*. Upregulated expression of TLR2 and TLR5, adaptor MyD88 and cytokines (IL-1β, IL-8 and TGF-‧1) was observed in fish fed the viable SE6, while elevated expression of TLR2, but not MyD88 and cytokines, was observed in fish fed the heat-inactivated SE6, which suggested that MyD88-independent TLR2 signaling pathway may be involved in the probiotic recognition in *E. coioides*. The induced activation of intestinal mucosal immunity, especially the enhanced expression of antibacterial epinecidin-1 and IgM, was consistent with the intestinal microbial data indicating that several bacteria were suppressed to undetectable levels by both the viable and heat-inactivated SE6.
Vagococcus spp.	3	**3** Refs: 9214 10782 9213	*V. fluvialis* stimulated leucocytes of sea bream and sea bass (dose-dependent). *V. fluvialis* is able to increase respiratory burst and peroxide content in sea bream leucocytes. *V. fluvialis* could be used as probiotic bacteria to protect sea bass against infection by *V. anguillarum*. *Vagococcus* showed the ability to compete for attachment site with V. anguillarum. Pro-inflammatory cytokines (IL-1, TNF-α and COX-2) become up-regulated following exposure to probiotic strain, suggesting that *V. fluvialis* L-21 induces an early inflammatory response in HK leucocytes.
Clostridium spp.	2	**2** Refs: 10761 8170	Supplementation of *C. butyricum* can mediate the humoral immune responses (the phagocytic activity of head kidney leucocytes, the total Ig level in serum and gut mucus, serum phenoloxidase activity and acid phosphatases activity were increased), improve growth performance, and enhance disease resistance in fish.
Vibrio spp.	6	**2** Refs: 10 9021	*Vibrio fluvialis* PM 17 did not engender any desired probiotic effects.
Pseudomonas spp.	5	**2** Refs: 6028 10	Intestinal epithelial cell (IEC) cultures exposed to live or heat-inactivated forms of *Pseudomonas* GP21 had a stimulated expression of g-type lysozyme, hepcidin, transferrin, and metallothionein. Probiotic effects of *Pseudomonas spp*. PM 11 were not observed in shrimp.
Candida spp.	2	**2** Refs: 10048 8406	Prawns fed with *Candida tropicalis* improved performance, decreased Nitro blue tetrazolium (NBT), and increased alkaline phosphatase activity after infection with White Spot Virus. *C. tropicalis* helps to enhance disease resistance through stimulation of the nonspecific immune system of the prawns. Enhanced disease resistance resulted in delayed and reduced mortality in prawns. *Candida parapsilosis*is supplemented in fish diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.

Arthrobacter spp.	2	**2** Refs: 6012 12712	*Arthrobacter spp.* CW9 improved the immune responses of *P. vannamei* against *V. alginolyticus* by increasing phenoloxidase activity, phagocytic activity, and clearance efficiency. The *Arthrobacter* strain had a restricted inhibitory spectrum but greatly inhibited *V. Anguillarum* in *Gadus morhua L.*
Aeromonas spp.	7	**1** Refs: 4123	*A. bivalvium* could promote growth, resist challenge to *V. harveyi,* and stimulate several immune responses (enhancement of both respiratory burst and superoxide dismutase activities, increase acid phosphatase activity of shrimp).
Aspergillus spp.	2	**1** Refs: 5426	The fermentation process of soybean meal with *Aspergillus oryzae* did not seem to affect growth performance and feed utilisation but could enhance the absorption of phosphorus and non-specific immune responses in juvenile parrot fish.
Streptococcus spp.	1	**1** Refs: 8313	In the shrimp treated with probiotic culture *Streptococcus phocae* PI80, upregulation of immune genes pro-phenoloxidase, serine protein (SP), β-1,3-glucan-binding protein and peroxinectin (PE) were observed. This upregulation coincides with enhanced prophenoloxidase activity as well as phagocytic activity and enhanced survival in probiotic-treated shrimps.
Escherichia spp.	1	**1** Refs: 9021	*E. coli* MG1655 F9 was able to colonise zebra fish larvae better than wild-type MG1655, indicating that protection of *E. ictaluri* infected larvae was correlated with the ability of MG1655 to colonise zebra fish. *E. coli* MG1655 F9 adhesion capacity provided by the F-plasmid and, to a lesser extent, type 1 fimbriae, is involved in the protection against *E. ictaluri* infection.
Alteromonadaceae spp.	1	**1** Refs: 2410	The use of two strains (Pdp11 and Pdp13) from the *Alteromonadaceae* family as probiotics had positive effects on the growth and intestinal functionality of juvenile *S. senegalensis*. *Alteromonadaceae* increased lysozyme activity. The lamina propria indicated signs of an inflammatory process, such as thickening, and increased the presence of leukocytes. Microvilli also seemed to be larger and more numerous, with normal apical borders and epithelial integrity.
Halomonas spp.	1	**1** Refs: 12897	Probiotic *Halomonas* spp. B12 could modulate the intestinal microflora and stimulate shrimp immunological levels and consequently enhance its effectiveness in preventing White Spot Syndrome Virus WSSV infections in shrimp. *Halomonas* diet increased total bacterial counts and decreased Vibrio spp. counts. Hemocyte counts, phenoloxidase (PO) activity in plasma and hemocyte lysate supernatant in the shrimp fed diets supplemented with probiotic B12 were significantly higher ($P<0.05$) than the control group. Mortality was reduced.
Phaeobacter spp.	1	**1** Refs: 2257	*Phaeobacter gallaeciensis* reduced the mortality of *V. Anguillarum* challenged cod larvae (*Gadus morhua*) to 10%, significantly below the levels of both the challenged and the unchallenged larvae.
Paffia spp.	1	**1** Refs: 10183	Even though no clear immunostimulatory effect could be identified, *Phaffia* diet had a positive effect on the animals, leading to better survival.

Hanseniaspora spp.	1	**1** Refs: 6602	*Hanseniaspora opuntiae* C21 improved phagocytic activity in the coelomocytes of sea cucumbers. C21 administration significantly enhanced lysozyme (LSZ), phenoloxidase activity (PO), total nitric oxide synthase (T-NOS), superoxide dismutase (SOD), alkaline phosphatase (AKP), acid phosphatase (AcP) activities in coelomic fluid, and LSZ, T-NOS, AKP and AcP activities in coelomocytes lysate supernatant (CLS) of sea cucumbers. *H. opuntiae* C21 can improve disease resistance against *V. splendidus* as well as tolerance to salinity stress by enhancing the immunity of the sea cucumber.
Zooshikella spp.	1	**1** Refs: 5401	The innate immune parameters, such as superoxide anion production, phagocytic and lysozyme activity, were enhanced after the eighth week of the *Zooshikella* diet. The enhancement of innate immune parameters by *Zooshikella* supplementation diets are possibly an important factor in reducing the percentage mortality and thereby protecting the *Paralichthys olivaceus* against live *S. iniae*.

3.4.5.2 Fish – prebiotics

3.4.5.2.1 Salmonids

Table 34: Mode of action of different prebiotics in fish (Salmonids)

Salmonids - Prebiotic	Number of articles		Mode of action
	Fish	Salmonids	
Glucan	31	**8** Refs: 1813 10639 2684 13169 11918 12042 11917 8939	Glucans act at the cellular (macrophage) membrane level via a specific receptor, and it may be possible that the linkage of glucan particles to specific receptors modifies the structure of the membrane in such a way that the macrophage becomes more efficient in the function of antigen processing and presentation. β-glucan bath exposure could be applied in the enhancement of innate immune factors by up-regulating the expression of complement factors (C3 and factor B) and acute phase proteins (hepcidin, precerebellin and transferrin). β-glucan induces an extracellular matrix remodelling process and leukocyte movement in both the gills and intestine. The MHC-II alpha chain was significantly up-regulated, suggesting a role for adaptive immunity after dietary immunostimulation in fish. Functional groups, including inflammatory response (stimulation of the production of pro-inflammatory cytokines and chemokines), response to bacteria, response to unfolded protein, and response to biotic stimulus, were reduced in the intestine, whereas detection of abiotic stimulus was induced. β-glucan influences the specific responses to an antigen in terms of antibody responses to vaccination. Lymphocyte proliferation in the presence of Concanavalin A, which induces the proliferation of T-cells, was also enhanced by glucan.
Mannan oligosaccharide (MOS)	25	**3** Refs: 13169	MOS modulates intestinal microbial communities, which subsequently improves gut morphology and the epithelial brush border. Administration of MOS up-regulated lysozyme and TNF-α gene and

		8939 2613	down-regulated HSP70 gene expression. Also, dietary Immunogen increased humoral immune response that necessarily resulted in higher resistance to the disease challenge test. It has been suggested that MOS stimulates mannose binding lectin (MBL) by liver secretion, binding the capsule of bacteria and triggering the complement cascade.
Fructooligosaccharide (FOS)	15	**2** Refs: 13095 7117	In these studies, FOS is supplemented in fish diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Yeast cell wall (YCW)	10	**1** Refs: 8597	Yeast extract inclusion in feeds was able to successfully induce inflammatory gene expression (IL-1β) in the head kidneys of infected fish with *L. salmonis*.
Lipopolysaccharide (LPS)	2	**1** Refs: 4005	LPS of *A. salmonicida* is reported to be highly immunogenic in Atlantic salmon. LPS is an activator of complement. As the fry received high doses of LPS each day, LPS could deplete complement components, leaving the fish more vulnerable to infection. LPS may have beneficial effects on the immune system of fry in relation to combatting infection. It is necessary to adjust the doses of LPS as well as the length of the stimulatory period.
Peptidoglycan	1	**1** Refs: 1541	Feeding peptidoglycan (PG) enriched diets to rainbow trout resulted in up-regulation of many AMPs (antimicrobial peptides) in mucosal tissues (skin, gills, gut) and liver.

3.4.5.2.2 Freshwater fish

Table 35: Mode of action of different probiotic in fish (Freshwater fish)

Freshwater - Prebiotic	Number of articles		Mode of action
	Fish	Freshwater	
Glucan	31	**12** Refs: 9417 1231 3027 8558 3028 5823 7729 2842 5776 5775 8559 4912	β-glucan may affect the composition of the carp intestinal microbial communities. Positive effects on intestinal microvilli length and density were observed. β-glucan increased intraepithelial leucocytes in the anterior intestine, which may indicate a localised immune response. It stimulated CRP as well as complement responses to PAMPs immunological challenges. The expression of inflammatory-related genes (IL1β, IL10, TNF-α1, TNF-α2, CXCa and CXCb) were down-regulated for all genes analysed in the spleen, head kidney, and the mid gut tissues when feeding with β-glucan additives.
Mannan oligosaccharide (MOS)	25	**4** Refs: 9398 8914 2842 13213	Dietary supplementation with MOS had no significant effects on survival, and the effects on performance and feed efficiency are unclear. MOS had no effects on haematological parameters, with the exception of lymphocyte and eosinophil levels, which were significantly decreased and elevated, respectively, in fish.
Fructooligosaccharide (FOS)	15	4	Dietary supplementation of 2-3% FOS could increase

		Refs: 12877 10732 12541 13212	microvilli length. The increase in microvilli length may mean improved apical brush border integrity and absorptive surface area. FOS can modulate the innate immune responses of Caspian roach fry. Fish fed FOS had significantly greater serum total immunoglobulin, serum lysozyme activity, and serum alternative complement activity (ACH50). White blood cell (WBC) count and lymphocyte levels increased significantly in the group treated with 1% FOS. Additionally, the content of red blood cells (RBC) means that corpuscular volume (MCV), haemaotcrit, haemoglobin and lymphocyte increased in the fish fed on diet 1%. Serum lysozyme activity was enhanced significantly in fish fed on the diet supplemented with 1% FOS.
Yeast cell wall (YCW)	10	**6** Refs: 6255 6256 4220 12994 5766 4886	Yeast polysaccharides supplementation could affect blood monocytes, leukocytes phagocytic activity, and could improve gut morphology in catfish. Fish fed 1% and 2% brewer's yeast had significantly higher serum peroxidase level and extracellular superoxide anion production of head kidney macrophages than tilapia fed the basal diet. YCW administered in doses of 40 and 60 g/kg feed statistically significantly activated the metabolic activity (RBA) of blood phagocytes and pronephric macrophages. Barramundi macrophages have receptors that recognise different glucans and initiate the respiratory burst response.
Inulin	6	**1** Refs: 7466	Inulin is supplemented in fish diets as a mixture with other probiotic. Therefore, a specific mode of action could not be attributed to it.
Galactooligosaccharides (GOS)	3	**1** Refs: 4486	GOS improves growth performance and stress resistance, modulating intestinal microbiota by increasing lactic acid bacteria of Caspian roach fry
Arabinoxylan oligosaccharides (AXOS)	3	**3** Refs: 3535 3533 3534	A comparison of the effects of two different preparations of AXOS on Siberian sturgeon indicates that AXOS, with a higher degree of polymerisation, improves the immune responses of fish. This observed enhancement of immune responses is likely related to the changes of the hindgut microbiota communities and the subsequent enhancement of short-chain fatty acid production
Lipopolysaccharide (LPS)	2	**1** Refs: 4658	*A. salmonicida* LPS is supplemented in fish diets as a mixture with other products. Therefore, a specific mode of action could not be attributed to it.
Chitooligosaccharide (COS)	1	**1** Refs: 6346	*A. veronii* infected fish supplemented with chitosan oligosaccharides (COS), *B. coagulans*, or a combination of the COS and *B. coagulans* showed increased protection against infection and higher survival rates, which indicate enhanced immunity and disease resistance. Koi fed with diets supplemented with a combination of the COS and *B. coagulans* had the highest final weight, specific growth rate (SGR), total leukocyte count (WBC), respiratory burst activity, phagocytic activity, lysozyme activity, SOD activity of koi and disease resistance to *A. Veroni*, followed by groups fed with diets with *B. coagulans* and COS.
Inactivated yeast-bacteria	1	**1**	2% *S. cerevisiae* var. *ellipsoideus* can be used as a growth promoter and intestinal microbial modulator for beluga

		Refs: 4487	juveniles.
Xylooligosaccharide (XOS)	1	**1** Refs: 4489	Feeding white fish fry with 3% XOS increased skin mucus antibacterial activity and total protein levels. Total autochthonous intestinal heterotrophic bacteria and lactic acid bacteria significantly increased following XOS administration in diet, while no significant effects on intestinal morphology, growth performance, or diet utilisation were observed.
Levan	1	**1** Refs: 3980	The haemoglobin content, total leucocyte count, lysozyme activity, and NBT were increased with a dietary supplementation of levan at 1% or more in *L. rohita* juveniles.

3.4.5.2.3 Marine fish / shellfish

Table 36: Mode of action of different prebiotics in fish (Marine fish / Shellfish)

Other marine fish / shellfish - Prebiotic	Number of articles		Mode of action
	Fish	Other	
Glucan	31	**11** Refs: 4011 1506 9549 10986 6493 10183 1665 2046 6473 964 461	β-1,3/1,6-glucan modulates the immune response (increase of phagocytic activity and IL1-band decrease of IgM) and stimulates growth of the gilthead sea bream. In marron, the dietary supplementation with β-glucan is a significantly higher total haemocyte count (THC) and granular cells. There is a clear-cut elevation in the haematological parameters, such as plasma protein, haemocyte count, phenol oxidase activity, superoxide anion production, alkaline phosphatase, and acid phosphatase activities in *F. indicus* fed glucan-incorporated diet. Oral administration of β-glucan can enhance the production of cell activating factors in the haemocytes, increasing ProPO and phagocytosis in shrimp. β-glucan indirectly affected the response of the host to pathogens by reducing the level of the anti-inflammatory cytokine IL-10 and increasing IL-1β.
Mannan oligosaccharide (MOS)	25	**18** Refs: 2280 2281 11468 12891 9548 11466 11467 11469 11470 9551 2614 12975 202 1304 6473 12581	MOS supplementation significantly enlarged intestine folds height and width, further increasing the intestinal barrier. This quantified enlargement would be related to the lamina propria engrossment observed, as a result of the higher infiltrated eosinophilic granulocytes (ECGs) seen on posterior gut lamina propia of fish fed MOS diet. MOS dietary supplementation stimulates GALT in terms of higher infiltration of leucocytes, particularly lymphocytes in the gut mucosa in relation tothe possible activation of the eicosanoid cascade in the posterior gut. MOS incorporation at 0.4% significantly improved head kidney macrophages phagocytic activity and lysozyme activity. MOS increases expressions of IL-1β and IL-8, playing a key role in differentiation and proliferation of different immune cells.

		1347 9550	
Fructooligosaccharide (FOS)	15	**9** Refs: 12905 12908 13185 12975 202 1304 12581 4488 6254	FOS (0.50%) resulted in significantly higher TCC (total coelomocytes counts), PO activity (phenoloxidase), and might enhance phagocytosis in sea cucumber. FOS can significantly improve disease resistance by enhancing immunity as well as presumably modulating microflora in the sea cucumber's gut. Fish fed with diet supplemented with FOS had a significantly lower neutrophil oxidative radical production in red drum. FOS had a significant detrimental effect on anterior intestinal structures in red drum. Haemoglobin (Hb) concentration, leucocyte (WBC) levels, and the proportion of lymphocytes were significantly higher ($P<0.05$) in the 2% oligofructose fed fish. scFOS can alter GI microbial composition as well as enhancing THC and hemocyte respiratory burst in shrimp.
Yeast cell wall (YCW)	10	**3** Refs: 12786 1347 202	YCW improved the growth performance and intestinal mucus development of Japanese sea bass. YCW enhanced the immune response and cumulative survival after challenge with *A. veronii*. The increasing globet cell number could explain the phenomenon of high immune response and low growth performance. Grobiotic-A increased the microvillus height in red drum. In hybrid striped bass, Grobiotic-A increase the fold length of mid and posterior intestine and the enterocyte height of the mid intestine. Additionally, this tended to increase the activities of chymotrypsin and alkaline phosphatase and showed a significant decrease in α-amylase activity.
Inulin	6	**5** Refs: 1606 1609 956 1604 6550	The combination of *B. subtilis* and inulin exerts a negative effect on gilthead sea bream gut homeostasis. No immunostimulant effect of inulin on the innate immune system of gilthead sea bream was noted. Inulin increases the PO activity on *L. vannamei*. Inulin reduces the WSSV prevalence in shrimp with low viral load.
Galactooligosaccharides (GOS)	3	**2** Refs: 12975 1347	GOS had a significantly higher plasma lysozyme compared to fish fed diets supplemented with other prebiotics. GOS was able to alter the intestinal microbiota.
Transgalactooligosaccharide (TOS)	2	**2** Refs: 202 1304	TOS Increased the length of intestinal folds and the height of enterocyte and microvilli. An increase was notedinactivities of pepsin, aminopeptidase, trypsin, α-amylase, and both acid and alkaline phosphatases in Red Drum.
Chitin	1	**1** Refs: 2958	The administration of a chitin diet (25 or 50 g/kg) enhances sea bream immune activity through the non-specific modulation of haemolytic complement activity, leucocyte respiratory burst activity, and cytotoxicity.

3.4.5.3 Fish – plant extracts

3.4.5.3.1 Salmonids

Table 37: Mode of action of different plant extract in fish (Salmonids)

Salmonids - Plant Extract	Number of articles		Mode of action
	Fish	Salmonids	
Soybean derivatives	6	**1** Refs: 9335	Rainbow trout fed both soya diets had depressed weight gains and elevated feed/gain ratio. Cell counts indicated increased leukocyte cell numbers as well as increased concentrations of plasma proteins and immunoglobulin in the soya-fed fish. Increased neutrophil, monocyte, and macrophage activity, as assessed by several oxidative radical production and phagocytic index assays, were higher in the soya-fed fish, possibly indicating an inflammatory or hypersensitivity response
Allium derivatives	3	**1** Refs: 7845	Garlic increases RBC and WBC. The production of superoxide anion as a measure of the respiratory burst activity and lysozyme activity was significantly influenced by dietary garlic.
Thymus vulgaris derivatives	2	**1** Refs: 3584	Dietary thymol supplementation improved feed efficiency, but body weight gain was unaffected. Total anaerobe counts were lower and the *Lactobacillus* load increased. Activity of glutathione based enzymes, levels of lysozyme, total complement concentrations, and catalase activity were higher in phytogenic supplemented groups. Thymol supplementation reduced NO serum levels significantly.
Zingiber officinale	2	**1** Refs: 2789	Rainbow trout fed with a diet containing 1% aqueous extract of powdered ginger roots, for three weeks, exhibited a significant non-specific immune response. Phagocytosis and extracellular burst activity of blood leukocytes were significantly higher than those in the control group. The level of plasma proteins was higher in the group fed with 1% ginger extract.
Urtica dioica	2	**2** Refs: 2789 401	Stinging nettle enhanced extracellular respiratory burst activity and increased the total protein level in plasma. Stinging nettle increased bactericidal activity, lysozyme activity, and Haemaotcrit (Hct) and Hb values. Feeding rainbow trout with 1% stinging nettle for 14 days led to reductions in mortality after challenge with *Aeromonas hydrophila.*
Viscum album	1	**1** Refs: 2789	*Viscum album* enhanced extracellular respiratory burst activity and increased the total protein level in plasma. Phagocytosis and extracellular burst activity of blood leukocytes were significantly higher than those in the control group.
Magnifera indica	2	**1** Refs: 401	Feeding rainbow trout with 1% mango for 14 days led to reduction in mortality after challenge with *Aeromonas hydrophila.* There was significant enhancement in serum bactericidal activity, respiratory burst, and lysozyme activity in the treatment group compared to the control group. Use of mango led to the highest number of red blood and white blood cells.
Carvacrol	1	**1** Refs: 3584	Dietary carvacrol supplementation improved feed efficiency, but body weight gain remained unaffected. Total anaerobe counts were lower. The activity of glutathione-based enzymes, levels of lysozyme, total complement concentrations, and catalase activity were higher in

			phytogenic supplemented groups.

3.4.5.3.2 Freshwater fish

Table 38: Mode of action of different plant extract in fish (Freshwater fish)

Fresh water fish - Plant Extract	Number of articles		Mode of action
	Fish	Freswater fish	
Achyranthes derivatives	3	**3** Refs: 1637 1638 8868	Serum protein, globulin level, and myeloperoxidase activity were significantly higher in the *A. aspera* diet group. *A. aspera* stimulates both specific immunity and non-specific immunity in Indian major carp, *C. catla*.
Thymus vulgaris derivatives	2	**1** Refs: 3958	Feeding the fish with 1% of Thyme group significantly increased the phagocytic activity in the blood of the *O. mossambicus*. The level of respiratory burst activity and lysozyme activity were non-significant, and mortality was decreased.
Propolis	2	**1** Refs: 456	Propolis has been found to enhance growth, feed utilisation, immune responses, disease tolerance, and body composition in eel, *A. japonica*. Serum and mucus lysozyme activity were higher in propolis diet group.
Magnifera indica	2	**1** Refs: 9421	Dietary administration of mango kernel increases immunity and survival of fingerlings of rohu. Fish fed with mango kernel showed enhanced superoxide anion production, lysozyme, serum bactericidal, serum protein, and albumin compared with the control group. Mortality was decreased.
Oscimum sanctum	2	**1** Refs: 4141	*Oscimum sanctum* is supplemented in fish diet as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Curcuma longa derivatives	1	**1** Refs: 4141	*Curcuma longa* is supplemented in fish diet as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Astragalus derivatives	1	**1** Refs: 12821	*Astragalus* polysaccharides (APS) promote enhanced weight gain, specific growth rates, and improved feed conversion ratio. APS stimulated respiratory burst activity, enhanced the phagocytic activity, and induced lysozyme activity in Nile tilapia.
Sanguinaria canadensis	1	**1** Refs: 8906	Sanguinarine had a positive effect on tilapia growth performance, with no apparent effects toward carcass composition, hepatic function, or health status.
Trigonella derivatives	1	**1** Refs: 3958	The haematological indicators (WBC, RBC, PCV, neutrophils and monocyte) in *Oreochromis mossambicus* fed with a diet supplemented with fenugreek, significantly increased compared to control groups. Fish showed elevated phagocytic activity (especially in the case of neutrophils) and lysozyme activity were significantly increased. The mortality was reduced. The results indicate that supplementation of fish with fenugreek could improve the haematological and immunological properties and could also increase the survival rate after challenge with *S. iniae*.

Rosmarinus officinalis	1	**1** Refs: 3958	The haematological indicators (WBC, RBC, PCV, neutrophils and monocyte) in *Oreochromis mossambicus* fed diet supplemented rosemary, significantly increased compared to the control group. Fish showed elevated phagocytic activity (especially in case of neutrophils), which was significantly increased. The mortality was reduced. The results indicate that supplementation of fish with rosemary could improve the haematological and immunological properties and increase the survival rate after challenge with *S. iniae*.
Azadirachta indica	1	**1** Refs: 4141	*Azadirachta indica* is supplemented in fish diet as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Withania somnifera	1	**1** Refs: 10393	The results demonstrate that fish (*L. rohita*) fed with W*. somnifera* root showed enhanced NBT levels, Phagocytic activity, total Immunoglobulin level, and lysozyme activity compared with the control group. The survival ratewas higher in experimental diets. Dietary *W. somnifera,* at the level of 2 g/kg, showed significantly higher protection against *A. hydrophila* infection.
Raw fibre	1	**1** Refs: 4248	The number of eosinophils and neutrophils were higher in fish (*H. huso juvenile*) fed a commercial product with raw fibre. Lysozyme activity was significantly higher.

3.4.5.3.3 Marine fish / shellfish

Table 39: Mode of action of different plant extract in fish (Marine fish / Shellfish)

Marine fish / shellfish - Plant Extract	Number of articles		Mode of action
	Fish	Marine fish / shellfish	
Soybean derivatives	6	**5** Refs: 5612 3326 7380 2128 5426	In gilthead sea bream, respiratory burst activity of blood significantly decreased in the bioprocessed soy product groups, while stimulated activity did not indicate significant changes. Serum myeloperoxidase levels, and total bacteriolytic activity of complement, increased significantly at the highest dietary inclusion of bioprocessed soybean, compared to the control group, while serum lysozyme levels did not indicate significant changes. Some alterations in intestinal morphology were observed in the distal intestine. A significant increase in the number of granulocytes was observed after feeding zebra fish diets containing soy protein contents. These dietary components also induced the expression of genes related to the innate immune system, including myeloid-specific peroxidase as well as the complement protein and cytokines. Serum lysozyme activity was not significantly affected by dietary inclusion of vegetable oils (soybean oil). The cacteriolytic capacity of fish fed diets based on n-6 fatty acids rich in oil (soybean oil) was significantly lower. Vegetable oils did not affect basal expression of TNF-α and IL-1β genes in proximal intestine. However, in the head kidney, dietary inclusion of fish oilstended to raise basal levels of TNF-α gene expression. Complete replacement of dietary fish oil with soybean oil increased TNF-α and IL-1β expression in the intestine and head kidney at day three post-infection with *P. damselae*. Vegetable

			oils, as a single lipid source, may affect some immune responses and inflammatory processes. Dietary soy saponins did not affect sea bream growth performance but may improve feed utilisation. Histological alterations of the distal intestinal mucosa were observed but were not characterised as inflammatory responses. The fermentation process of soybean meal with *Aspergillus oryzae* does not affect growth performances and feed utilisation in parrot fish. We suggest that the fermentation process of soybean meal could enhance the absorption of phosphorus and non-specific immune responses (antioxidant activity, superoxide dismutase activity) in juvenile parrot fish.
Allium derivatives	3	**2** Refs: 7846 1865	Use of allicin led to a lower number of white blood cells compared to the levels in controls but elicited increased phagocytic activity, i.e. a phagocytic value of 39.2% compared to 13.6% in the controls, and serum lysozyme activity, which showed significant (P>0.05) differences compared to the control in *Oncorhynchyus mykiss*. Dietary inclusion of 0.5% onion powder was effective at improving lysozyme activity infish (olive flounder), and onion powder seemed to be an effective immunostimulant to lower mortality upon *E. tarda* infection.
Echinacea	2	**2** Refs: 7109 8406	*E. purpurea* has molecules with immunostimulant activity as caffeic acid derivatives, polysaccharide fractions, alkamides, quinovic acid glycosides, and pentacyclic oxindole alkaloids. The prevalence of shrimp infected with WSSV decreased in animals fed with Powder Plant. *E. purpurea* is supplemented in fish diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Uncaria tomentosa	2	**2** Refs: 7109 8406	*U. tomentosa* has molecules with immunostimulant acticvity as caffeic acid derivatives, polysaccharide fractions, alkamides, quinovic acid glycosides, and pentacyclic oxindole alkaloids. The prevalence of shrimp infected with WSSV decreased in animals fed with Powder Plant. *E. purpurea* is supplemented in fish diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Zingiber officinale	2	**1** Refs: 11192	The bioactive compounds identified in ginger directly affect fish health by activating the immune mechanism. The increase in WBC counts, neutrophils, and other blood cells following the feeding of a ginger diet, demonstrates the immunostimulatory effects and anti-infection properties of ginger in *Lates calcarifer* fish. The haemoglobin content, respiratory burst activity by neutrophils, and serum lysozyme were significantly higher.
Propolis	2	**1** Refs: 13083	The ethanolic-extract of propolis was more effective than the crude propolis in protecting fish (*Oreochromis niloticus*) from infection. Propolis enhanced the macrophage-functions and lymphocyte proliferation as well as resistance to several pathogens. The crude propolis and its ethanolic-extract significantly increased serum bactericidal activity against *A. hydrophila*. Propolis-ethanolic-extract and crude propolis significantly increased the serum lysozyme activity and the HCT-level.
Oscimum sanctum	2	**1** Refs: 8404	*Oscimum sanctum* is supplemented in fish diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Other Chinese herbs	1	**1** Refs:	*Scutellaria baicalensis* administered alone increases lysozyme activity. Administration of the *Scutellaria baicalensis* + probiotic enriched diets helps to restore the altered haematological values due

		4144	to infection and enhance the innate immune response and disease resistance against *E. tarda* in *O. Fasciatus.*
Glycyrrhiza	1	**1** Refs: 4145	*Glycyrrhiza* is supplemented in fish diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Plantago derivatives	1	**1** Refs: 2871	*Plantago asiatica* is supplemented in fish diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Citrus by product	1	**1** Refs: 6075	The fermentation of *Citrus* by-products with probiotics could have beneficial effects on innate immunity and thereby increase survival and disease resistance in juvenile olive flounder. Myeloperoxidase and lysozyme activities were increased in a dose-dependent manner by dietary *Citrus* by-products-*Bacillus subtilis* inclusions. In a consecutive challenge test against *E. tarda*, increased disease resistance was identified.
Carthamus	1	**1** Refs: 2871	*Carthamus tinctorius* is supplemented in fish diet as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Linum usitatissimum	1	**1** Refs: 7380	Vegetable Oils (linseed oil) did not affect basal expression of TNF-α and IL-1β genes in the proximal intestine. However, in the head kidney, dietary inclusion of vegetable oils tended to raise basal levels of TNF-α gene expression but did not affect IL-1β expression. Fish oil substitution by linseed oil increased lysozyme activity in infected fish and promoted bactericidal activity. Complete replacement of dietary fish oil with Linseed oil increased IL-1β expression in the intestine and head kidney post-infection with *P. damselae*. Vegetable oils, as a single lipid source, may impact some immune responses and inflammatory processes.
Mentha piperita	1	**1** Refs: 11191	The main active component of *M. piperita* leaves is the essential oil that contains a number of bioactive compounds (such as phenolic, tannins and flavonoids), which have been known to exhibit biological activities, especially antimicrobial ones. This study demonstrated higher phagocytosis in treated groups, which clearly indicates that the addition of *M. piperita* leaves in the diet significantly improved the nonspecific immunity of fish. *M. piperita* has been considered to have effective antioxidant and antiperoxidant properties, which trigger the production of the superoxide anion quantified by the nitroblue tetrazolium (NBT). The higher anti-protease activity protected the fish from infection and, as a result, fish showed resistance to the pathogen which thus increased survival.
Panax ginseng	1	**1** Refs: 4145	Panax ginseng is supplemented in fish diets as a mixture with other probiotics and herbs. Therefore, a specific mode of action could not be attributed to it.
Rubus coreanus	1	**1** Refs: 10985	*R. coreanus* ethanolic extract (RcEE) is a potent herbal immunostimulant. The diet enriched with RcEE could enhance SOD, CAT, GPx, AKP and ACP activity and decreased MDA activity with increased mRNA expression of cytosolic-SOD, CAT, GPx, which resulted in enhanced immunity in *P. vannamei*.
Alnus firma	1	**1** Refs: 4147	Dietary supplementations with *A. firma* protected the haematological profile, enhanced the immune parameters, and decreased the mortality. WBC count, haemoglobin and haemaotcrit levels, respiratory burst activity, and lysozyme activity were increased after two weeks of the *Alnus firma* diet administration in olive flounder fish.

Cucurbita	1	**1** Refs: 2871	*Cucurbita moshata* is supplemented in fish diets as a mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Lonicera	1	**1** Refs: 2871	*Lonicera japonica* is supplemented in fish diets as mixture with other herbs. Therefore, a specific mode of action could not be attributed to it.
Kalopanax pictus	1	**1** Refs: 4142	*K. pictus*-supplementation diets positively protected and enhanced the immune system in kelp grouper *E. bruneus* against *V. alginolyticus* and *P. dicentrarchi* infection. The red blood cells (RBC), white blood cells (WBC), haemoglobin, haematocrit, lymphocytes, and monocytes levels significantly increased in kelp grouper fed with all doses of *K. pictus*-supplementation diets and challenged with bacterium and parasites when compared to control groups. The respiratory activity, lysozyme activity, bactericidal activity, total protein level, and myeloperoxidase levels significantly increased in kelp grouper fed with all the doses of *K. pictus*-supplementation diet and challenged with bacterium and parasites.
Lactuca indica	1	**1** Refs: 4143	*L. indica* extract stimulates the immunological parameters and increases disease resistance in *E. bruneus* against *S. iniae* infection. The NBT level, phagocytic activity, and lysozyme activity increases after L. indica diet administration in *E. bruneus*.
Garcinia mangostana	1	**1** Refs: 10773	Feeding *Clarias gariepinus* with mangosteen (*Garcinia mangostana L.*) extracts showed no adverse affect on growth but enhanced the hematological parameters. Significantly higher red blood cell (RBC) count and white blood cell (WBC) counts were recorded. The erythrocyte count increased with the administration of mangosteen, which might indicate an immunostimulant effect.
Aconitum koreanum	1	**1** Refs: 4145	*Aconitum koreanum* is supplemented in fish diets as a mixture with other probiotics and herbs. Therefore, a specific mode of action could not be attributed to it.
Phoenix dactylifera	1	**1** Refs: 2957	The results showed that experimental diets altered the expression of SOD, CAT, and glutathione reductase (GR), particularly in the gill and skin. Palm fruit extracts may serve as good natural antioxidants.
Broussonetia kazinoki	1	**1** Refs: 5402	Phagocytic, complement, lysozyme activities, and respiratory burst were significantly enhanced by *B. kazinoki* diets. *B. kazinoki*-supplementation diets positively enhanced the innate immune system against *S. parauberis* in *P. olivaceus*.

3.4.5.4 Fish – animal by-products

3.4.5.4.1 Salmonids

Table 40: Mode of action of different animal by-products in fish (Salmonids)

Salmonids – Animal by-product	Number of articles		Mode of action
	Fish	Salmonids	
Lactoferrin	3	**1** Refs:	Effects on non-specific immunity or disease resistance after feeding Atlantic salmon lactoferrin (LF) supplemented diets were detected.

		6587	

3.4.5.4.2 Freshwater fish

Table 41: Mode of action of different animal by-product in fish (Freshwater fish)

Freshwater fish – Animal by-product	Number of articles		Mode of action
	Fish	Fresh water fish	
Lactoferrin	3	**1** Refs: 5824	LF supplements, particularly at the 100 mg level, enhanced serum lysozyme level, oxidative radical production, and the level of protection against *A. Hydrophila* in non-vaccinated *Clarias batrachus*. LF is an important component ofthe microbicidal activity of leucocytes.
Other	1	**1** Refs: 8750	Oral administration of chito-oligosaccharides/chitosan-oligosaccharides from shrimp significantly reduced the inflammatory response and stress response in tilapia intestine, as evidenced by decreased levels of mRNAs encoding, TNF-α and HSP70, and increased TGF-β.

3.4.5.4.3 Marine fish / shellfish

Table 42: Mode of action of different animal by-product in fish (Marine fish / Shellfish)

Marine fish / shellfish - Animal by-product	Number of articles		Mode of action
	Fish	Marine fish / shellfish	
Lactoferrin	3	**1** Refs: 2959	LF has a direct but weak effect on sea bream leucocytes and, when added to a common fish diet, activates the cellular gilthead sea bream's innate immune response by enhancing respiratory burst and leucocyte-mediated cytotoxic function, particularly at 100 mg/kg.
Antibodies	1	**1** Refs: 12514	IgY egg powders can be formulated into any type of feed. Abalone-fed anti-*Vibrio* IgY indicates higher respiratory burst activity; most importantly, it afforded prevention from *V. alginolyticus* infection.
Other	2	**2** Refs: 4997 5703	Fish protein hydrolysates (FPH) may promote early development of cod larvae. The key parameters (IgM and lysozyme) of the innate immune system of larvae were stimulated and increased. Different molecular weight fractions of FPH and concentrations of feed-soluble peptides may affect the growth performance and immunological status of sea bass larvae.

3.4.5.5 Fish – other substances

3.4.5.5.1 Salmonids

Table 43: Mode of action of other substances in fish (Salmonids)

Salmonids - Others	Number of articles		Mode of action
Substance / Agent	Fish	Salmonids	
Vitamins	20	**4** Refs: 6587 11917 11918 1961	Under the experimental conditions used, the combination of glucan and high doses of vitamin C, for a short feeding period, had a stimulatory effect on the non-specific and specific immune parameters tested. Vitamin C enhanced non-specific immune parameters, such as phagocyte activities and lysozyme levels, while β-1,3/1,6 glucan influenced specific responses to an antigen in terms of antibody response in *Oncorhynchus mykiss*. High dietary levels of vitamin E modulate the phagocytic functions of gut leucocytes in rainbow trout. The vitamin E diet effect seems to be greater inthe local intestinal response compared to systemic (head kidney) response.
Algae	17	**2** Refs: 4247 10413	Ergosan (*Laminaria digitata* and *Ascophyllum nodosum*) enhanced the density of the goblet cells in fish intestine and pyloric caeca despite as light increase in fish villus length and fold length in the intestine and pyloric caeca shown in the *Oncorhynchus mykiss*. Ergosan increased villus, fold, and enterocyte height and augmented the surface area of the gut mucosa. Haematological parameters (total white blood cells and red blood cells) showed a significant increase in the Ergosan-treated group when compared with the control group. Ergosan also increased lysozyme activity in the skin mucus, protease activity, and alkaline phosphatase activity. Ergosan enhanced the major immune components of rainbow trout mucus involved in the non-specific immunity
Others	7	**1** Refs: 8597	CpG ODN inclusion in feed was able to successfully induce inflammatory gene expression (IL-1β) in the head kidneys of fish infected with *Lepeophtheirus salmonis*.
Minerals	6	**1** Refs: 8597	Organic minerals are supplemented in fish diets as a mixture with other prebiotics, fed as a commercial product. Therefore, a specific mode of action could not be attributed to the minerals.
Organic acids derivatives / Fatty acids	4	**1** Refs: 3813	Tetradecylthioacetic acid (fatty acid) TTA have anti-inflammatory effects on the Atlantic salmon kidney (ASK) cell line (increased expression of anti-inflammatory IL-10). TTA attenuates the capacity for production of pro-inflammatory PGE2 and increased the expression of Arg1. It also modulates the immune system of Atlantic salmon.
Nucleotides	3	**1** Refs: 1349	Supplementation of fish feeds with exogenous nucleotides can have a positive influence on gut structure, disease resistance, immune response, stress tolerance, vaccine efficacy,and osmoregulatory capacity at transfer and growth rates in salmon.

3.4.5.5.2 Freshwater fish

Table 44: Mode of action of other substances in fish (Freshwater fish)

Fresh water fish - Others	Number of articles		Mode of action
Substance / Agent	Fish	Fresh water fish	
Vitamins	20	**12** Refs: 4912 12334 7666 9419 5821 11329 7379 12524 13208 8426 3103 3104	Application of glucan and vitamin U in the diets of fish modulated the non-specific defence mechanisms in sturgeon. Vitamin C, in both the non-specific and specific immune responses of rohu, increased serum parameters, respiratory burst activity, and antibody titers. High dietary vitamin C enhanced the non-specific immunity of fish, including an enhanced phagocytic ratio, bactericidal activity, respiratory burst activity, and increased serum lysozyme activity. The influence of Ascorbic Acid on immune response may be related to its antioxidant activity as a free radical scavenger, protecting cells from auto-oxidation and maintaining their integrity for optimal functioning of the immune system. Supplementation of vitamin E in fish diets seems to protect the complement system from stress-related reduction of activity. Dietary choline enhanced the serum activities of lysozyme and ACP, haemagglutination titer, C3 and C4 levels, and the leucocytes phagocytic activity of juvenile Jian carp after challenge; this suggests an improvement of the innate immune response of juvenile Jian carp, caused by choline. IgM and anti-*A. hydrophila* antibody titer in serum was enhanced by choline, indicating that choline also improved the specific immune responses of juvenile Jian carp. In Pyridoxine-fed (vitamin B6) group the erythrocytes count, haemoglobin content and total serum protein, albumin, globulin, nitroblue tetrazolium and lysozyme activity were significantly increased while cortisol and blood glucose were decreased. Dietary supplementation of pyridoxine mitigates endosulfan-induced stress and helps to modulate immunity. In Pantothenic acid (vitamin B5), Leucocyte phagocytic activity, lectin potency, lysozyme activity, and acid phosphatase activity, as well as the total iron-binding capacity, were improved. Serum immunoglobulin M levels and agglutination antibody titer to *A. hydrophila* were also increased. Pantothenic acid also promoted the growth and reproduction of Lactobacillus while depressing *Escherichia coli* and *A. hydrophila* in juvenile Jian carp.
Algae	17	**6** Refs: 12688 4248 4858 47 4658 3638	The *Chlorella* could increase the total protein (TP), albumin, and globulins in the serum of gibel carp. Additionally, the Chlorella could increase the superoxide dismutase (SOD) and lysozyme activity. Ergosan has stimulatory properties on cytokine gene expression involved in innate immune response: liver IL-1β, IL-8 and TNF-α2 gene expressions were significantly higher in trout fed on Ergosan compared with controls, indicating a positive role of this feed additive with regard to improving the immune responsiveness to the AquaVacTM vaccine. Gene expression of Hsp70, and plasma cortisol levels, were lower in Ergosan-treated *Oncorhynchus mykiss*. Ergosan generally has a positive effect on growth performance, lysozyme activity, Lymphocyte count, and neutrophil count in beluga (*H. huso*) juveniles. *Dunaliella salina*, as a food additive, can positively impact growth, immunological, and haematological parameters of *H. severus*. WBC,

			RBC, PCV and Hb were significantly increased and mortality was significantly decreased.
Others	7	**5** Refs: 1816 5822 10381 10999 4658	**Extracellular extracts** of *Aeromonas veronii* and *Flavobacterium sasangense* cultures increased the innate immune parameters of carp. The expression of three immune-related genes (IL-1β, TNF-α, Lysozyme-c) in the blood was significantly up-regulated and enhanced carp's resistance to *A. hydrophila* infection. **Levamisole** supplement, at the lowest level (50 mg/kg), significantly enhanced oxidative radical production and serum myeloperoxidase (MPO) content immediately after 10 days of feeding in Asian catfish. Haemolytic complement and haemagglutination titer were significantly enhanced. Fish fed diets supplemented with **Tribasic copper chloride** showed significantly increased T-AOC activity and relatively higher survival rates. Fish fed diets supplemented with Tribasic copper chloride showed higher activity of AKP and ACP. **Poly-b hydroxybutyrate-hydroxyvalerate** (PHB-HV) from *Bacillus thuringiensis* supplemented diet were found to have significant immunostimulatory effects on both specific and nonspecific immunity (antibody response, lysozyme activity, number of neutrophils), which in turn impacted functional immunity in terms of disease resistance against live virulent *A. hydrophila* in tilapia. **Yeast DNA** induced changes in B-cell kinetics in PBL and the head kidney, suggesting suppressive effects that strongly indicate oral or juvenile tolerance in carp.
Minerals	6	**3** Refs: 13172 1577 990	Dietary **Chromium** supplementation enhanced carbohydrate utilisation and growth performance attributed partially to the activation of liver key enzymes (HK and G6PD) in mirror carp. Tilapia supplemented with Cr presented with increased total numbers of cells at inflammatory focus, with significant accumulation of lymphocytes and reduction in cortisolemia and glycaemia. Dietary **activated charcoal** tended to increase the height of intestinal villi and goblet cell number in Nile tilapia.
Amino acids and derivatives	6	**4** Refs: 11220 5758 12329 1801	Dietary **methionine** levels significantly promoted plasma lysozyme activity, lectin potency, and C3 and C4 contents in juvenile Jian carp. Blood serum sim-IgM gradually increased with methionine supplementation. **Methionine hydroxyl analogue (MHA)** improved disease resistance by increasing the growth of immune organs and humoral immune response in Jian carp. MHA depressed lipid or protein oxidation in head kidney and spleen to protect structure and function of immune organs. MHA affects antioxidant enzyme activity and GSH content in the head kidney and spleen, thus improving immune response. Dietary **Tryptophan** Trp improved fish growth, intestinal innate immunity, barrier function, and antioxidant status while attenuated the intestinal inflammatory response of young grass carp. Dietary Trp exerted an anti-inflammatory effect by upregulating the IL-10 and TGF-β1 gene expression and downregulating the TNF-α and IL-8 gene expression; this may be related to the down-regulation of the target of rapamycin (TOR) in the intestines of fish. Trp up-regulated the gene expression of occludin, ZO-1, claudin-b, -c, and -3, and down-regulated the gene expression of claudin-12 and -15; this improved the intestinal barrier function of fish. Dietary Trp enhanced the SOD1 and GPx activities and mRNA levels in the intestines of fish, and this may be associated with the Nrf2- Keap1 signalling pathway. **Arginine** and/or **Glutamine** enhanced final weight, specific growth rate, and feed efficiency in hybrid striped bass (HSB). Dietary Arg and Gln supplementation tended to improve neutrophil oxidative radical production and intracellular superoxide anion production by HSB kidney

			macrophages. Arginine and/or Glutamine increased lysozyme activity, extracellular superoxide anion, fold height in the mid- and distal intestine, and microvillus height in the pyloric caeca.
Organic acids derivatives / Fatty acids	4	**1** Refs: 3754	Silage oil improved cellular non-specific immunity and simultaneously decreased total mortality in tilapia. Silage oil also exhibited significant antimicrobial effects in the feed and GIT of experimental fish. The reason for the improved phagocytic activity of leukocytes in fish fed the silage oil diet could be related to improvement in dietary fatty acid balance.
Enzymes	2	**2** Refs: 5816 1484	Diets supplemented with **Amylase enzyme** increased WBC count, total plasma protein, serum globulin, and respiratory burst activity in *L. rohita* juveniles. Supplementation of AiiAB546 (**N-acyl Homoserin Lactonase** from *Bacillus spp*. B546) into fish diets influenced gut microbiota and attenuated bacterial pathogenicity. The higher expression levels of IFN-γ might be derived from the reduction of virulent factor expression of *A. hydrophila*. The expression levels of IL-10, TLR5b, and iNOS2a were significantly increased in the enzyme group than the levels in other experimental groups. This phenomenon might be due to the contribution of the yeast polysaccharide residues present in recombinant AiiAB546, which function as immunity activators in feed additive.
Peptides	2	**1** Refs: 12982	Feeding common carp with **apidaecin** could improve growth performance and induce a positive modulation of immune response, likely through regulation of the intestinal microflora, inhibition of the growth of gram-negative potential pathogens, and stimulation of the growth of intrinsic probiotics. Apidaecin could increase the serum lysozyme activity of common carp, and the effect was concentration-dependent.

3.4.5.5.3 Marine fish / shellfish

Table 45: Mode of action of other substances in fish (Marine fish / Shellfish)

Marine fish / shellfish – Others	Number of articles		Mode of action
Substance / Agent	Fish	Marine fish / shellfish	
Vitamins	20	**4** Refs: 2172 9238 6129 8041	Vitamin C is a beneficial dietary supplement often used for improving growth performance and elevating mucosal immune response (skin mucus protein levels and alkaline phosphatase activity) in Caspian roach fry. *P. monodon* fed diets containing ascorbate demonstrated that vitamin C is an immunostimulant for *P. monodon*. Increased weight gain (WG), feed efficiency (FE), survival, total haemocyte count (THC), superoxide anion (O2-), production ratio, and phenoloxidase (PO) activity were observed in shrimp fed diets supplemented with vitamin C. High dietary doses of vitamin E (α-tocopherol acetate), together with an adequate vitamin E / vitamin C ratio, may increase the gilthead sea bream's non-specific immune response by enhancing serum complement activity as well as phagocytosis-mediated leucocyte function. Serum IgM levels infish fed with the assayed vitamin A-supplemented diets were statistically higher than those in fish fed a non-supplemented diet, for sea bream.
Algae	17	**9** Refs:	Dietary administration of alginic acid (Ergosan) had a significant effect on serum complement activity in sea bass. In shrimp, differential analysis of haemocyte populations revealed

		461 1608 9048 1607 1605 10617 4582 9051 7382	marked changes in terms of relative levels of hyaline, semi-granular, and particularly granular haemocytes in diets supplemented with Ergosan. Microalgae *N. gaditana*, *T. chuii* and *P. tricornutum* can stimulate certain activities inthe gilthead sea bream's immune system and can slightly affect immune-related gene expression. *Phaeodactylum tricornutum* generally hasan increased enhancing effect on the immune parameters of gilthead sea bream. The effect of this microalga might be attributed to the presence of a cell-wall-sulphated glucomannan and a β-1,3-glucan. Microalgae increased the expression of CSF-1R and MHC-IIa genes in the gut. Dietary applications of *B. subtilis*, *T. chuii*, and *P. tricornutum*, either singly or in combination, may exhibit up-regulating effects on gilthead sea bream immune parameters (complement activity, IgM level, respiratory burst, and phagocytic activity). Fish (*Lutjanus peru*) fed with microalgae Navicula + *L. sakei* increased myeloperoxidase, lysozyme, total antiproteases activities, and IgM. Shrimp fed the *Gracilaria tenuistipitata* extract-containing diets showed increased immunity through increasing circulating haemocytes, PO activity, RBs, SOD activity, GPx activity, and lysozyme activity along with increases in mitotic cells and the mitotic index of HPTs, which indicated increased resistance to *V. alginolyticus* and WSSV infections. The shrimp treated with *Sargassum fusiforme* polysaccharide extract (SFPSE) displayed significantly lower cumulative mortalities and enhanced immune activity (THC, PO and LSZ activity).
Others	7	**1** Refs: 12906	**Isomaltooligosaccharide (IMO)** is supplemented in fish diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Minerals	6	**2** Refs: 10485 9088	**Barodon (Anionic Alkali Mineral Complex)** supplementation affects the membrane-associated lymphoid tissues. Innate immune response of olive flounder was upregulated by Barodon, indicating significantly increased lysozyme, MPO, and SOD activities. **Iron** supplementation enhanced the performance of gilthead sea bream.
Amino acids and derivatives	6	**2** Refs: 1800 8591	Supplementation with **Arginine** and/or **Glutamine** enhanced the feed efficiency of red drum as well as promoting the development of the intestine. Arginine and/or Glutamine increase neutrophil oxidative radical production, serum lysozyme activity, and extracellular and intracellular superoxide anion production. Morphometric (enterocyte, microvillus, and fold height) demonstrated the positive effects of both dietary glutamine and arginine, in different portions. Glutamine increased mucosal fold length and both enterocyte and microvilli height in all intestinal sections in channel catfish.
Organic acids derivatives / Fatty acids	4	**2** Refs: 233 1831	**Acidic Calcium Sulphate** increased the phagocytic activity in haemocytes and elevated total hemocyte counts. It increased stress tolerance, as indicated by significant effects on both hemolymph protein and glucose. The prawn *Penaeus stylirostris* (Stimpson), when fed for 28 days with **n-3 highly unsaturated fatty acid (HUFA)**-enriched feed pellets, demonstrated an improvement in immune defence potential (increased agglutination titer of plasma and increased respiratory burst of haemocytes).
Nucleotides	3	**2** Refs: 8384 1799	Nucleotide supplementation did not significantly influence the growth of fish fed diets with 30% to 50% soybean meal, but could be helpful in improving the non-specific immune response and the intestinal histological structure of turbot. Nucleotide supplementation tended to improve weight gain and survival of red drum, though not at a significant level. Dietary nucleotides supplemented at 1% of the diet enhanced the extracellular superoxide anion production of red drum kidney macrophages. Additionally, nucleotide supplementation increased the

			proximal intestine fold height and villus height.
Peptides	2	**1** Refs: 3620	Oral administration of immuno-stimulatory peptides does not provide efficient protection to fish fry when they are exposed to high levels of highly virulent pathogens.
Fungi - Mushroom	1	**1** Refs: 9179	Dietary administration of the fungus *M. circinelloides* in sea bream increases complement and lysozyme activity, lymphocyte mitogenesis, leucocyte phagocytosis, and the secretion of TNF-α, disease resistance and cell-mediated cytotoxicity. The mechanism by which they exert their influence may be their antioxidant and adjuvant properties.

3.4.6. Rabbits

3.4.6.1 Rabbits – probiotics

The most frequently examined microorganism related to probiotics has been *Saccharomyces cerevisiae*, which has been reported in three reviewed papers (Table 46). Only in one case was the *S. cerevisiae* used as the unique component; other microorganisms were discussed together in the two remaining articles.

In the paper where *Saccharomyces cerevisiae* was assayed alone, the amount of intestinal yeast was significantly increased, indicating a good survival rate during the feed pelleting process. However, no positive effects were obtained for productive parameters, carcass characteristics, caecal fermentation, and meat quality. No data on immunological response or intestinal mucosa morphology were examined, although this data could be useful considering the effect of the yeast wall on these parameters.

When *Saccharomyces cerevisiae* was used in combination with other microorganisms (*Enterococcus faecium* as in one paper, for example, or lactic acid bacteria, *Candida utilis, Streptomyces albus, Streptomyces griseus, Aspergillus oryzae* and *Mucor hiemalia* in another paper) no significant effects on intestinal microbiota or performance parameters were observed.

Bacillus spp. was examined in two papers. In the first, a combination of *Bacillus licheniformis* and *Bacillus subtilis* was used, resulting in significant reduction of mortality and decreasing the presence of *Escherichia coli* and *Clostridium perfringens* (two of the most important pathogens in rabbits) in faeces. Additionally, improved growth performance (ADG and FCR) was obtained after the use of these two *Bacillus spp*. In the second paper, the effects of *Bacillus cereus* were examined with another probiotic, *Pediococcus acidilactis*, and two

prebiotics, mannan oligosaccharides and arabinoxylans oligosaccharides. No significant positive effects were obtained in the group supplemented with *Bacillus cereus*.

Lactobacillus spp. is not a significant component of the digestive microbiota of rabbits, but the use of Lactobacillus paracasei as feed additive was examined in one paper. The inclusion of this resulted in positive effects on villi and crypts structure as well as reduction in intestinal damage and clinical signs after the experimental infection of rabbits with *Staphylococcus aureus*.

The role as probiotic of *Enterococcus faecium* CCM7420 strain has been analysed in one paper, with good results when applied alone or in combination with the prebiotic *Eleutherococcus senticosus*. The *Enterococcus faecium* CCM7420 strain improved performance, stimulated digestive immunity, and increased the host's defence, increasing phagocytic activity and reduction/competition with potential pathogens without induction of oxidative stress in rabbits. In another paper, the strain NCIMB 30183 of *Enterococcus faecium* was studied in combination with *Saccharomyces cerevisiae* NCYC Sc47 strain; effects on the intestinal amount of *Clostridium spiroforme*, *Bacteroides spp.* or *Fibrobacter succinogenes* was not observed in any experimental group, and only a significant increase of *Enterococcus faecium* in faeces was observed in the groups supplemented with the *Esterococcus* probiotic strain.

One paper examined *Bifidobacterium adolescentis* as a probiotic in rabbits. In this work, *Bifidobacterium adolescentis* improved the ileum mucosal microvilli height and the crypt depth, reduced the bacterial translocation rate and gut permeability, and also reduced the serum endotoxin levels.

Prediococcus acidilactis was used in combination with *Bacillus cereus*, mannan oligosaccharides, and arabinoxylans oligosaccharides in a previous paper. No significant positive effects were obtained in the group supplemented with *Prediococcus acidilactis*.

One paper studied the probiotic effects of *Lactoccocus lactis.* This acid lactic bacterium induced a significant decrease in the translocation of *Enterobacter cloacae* to the lung, the MLNs in the liver, and the spleen of rabbits. No other effects were examined in this study.

Table 46: Mode of action of different probiotics in rabbits

Rabbits - Probiotic	Number of articles	Mode of action
Saccharomyces spp.	**3** Refs: 9284 702 9506	Supplementation with *Saccharomyces* did not affect the productive performance, carcass characteristics, caecal fermentation, or meat quality of rabbits. *S. cerevisiae* did not influence the level of Bacteroides species, *F. succinogenes*, *E. faecium,* or *C. spiroforme* after 14 days of supplementation. However, an improvement in gut microbial population was observed by the synergistic action of yeast and fungi on beneficial lactic acid bacteria both in the caecum and the colon. This may be due to the provision of soluble nutrients, particularly B vitamins.
Lactobacillus spp.	**2** Refs: 703 9506	*Lactobacillus* enhanced the growth of lactic acid bacteria (LAB) in the rabbit caecum and colon. In addition, administration of milk containing *L. paracasei* subspp. *paracasei* to rabbits infected with multidrug resistant *S. aureus* arrests diarrhoea and improves the reestablishment of the intestinal and colonic mucosae.
Bacillus spp.	**2** Refs: 5744 8053	Growth performance characteristics (ADG and FCR) were improved by the supplementation of rabbit diets with *Bacillus*. The main potential pathogens, such as *E. coli* and *C. perfringens*, were reduced in rabbits following probiotic treatment. Probiotics could reduce the adhesion of these pathogens to mucus.
Enterococcus spp.	**1** Refs: 10571	Diet supplementation with *E. faecium* CCM7420 could improve growth performance, stimulate digestive immunity, and increase the host's defence capacities by increasing phagocytic activity and reduction/competition with potential pathogens (decrease of faecal coliforms, *Pseudomonas*-like *spp.*, *Clostridium*-like *spp.* and *S. Aureus*) without induction of oxidative stress in rabbits. The reduction of *Enterococci* and lactic acid bacteria can likely be explained by the activity of the strain against other enterococci and/or LAB. No negative influences of probiotic application on GPx values werereported.
Bifidobacterium spp.	**1** Refs: 12519	Rabbits administered with *Bifidobacterium* demonstrated obvious improvement in the degree of gut atrophy and extent of liver injury; bacterial translocation, gut permeability and serum endotoxin levels were reduced when compared with rabbits fed with parenteral nutrition without the supplementation of *Bifidobacterium*. *Bifidobacterium* feeding maintained the ileum mucosal villus height and the crypt depth and reduced gut permeability. Therefore, the gut barrier was reinforced, thereby reducing the number of pathogenic bacteria/endotoxin translocating from the gut to the liver. The decreased concentration of serum endotoxin in the *Bifidobacterium* group also suggested a decreased level of systemic inflammation response, which was related to the alleviation of liver injury.
Pediococcus spp.	**1** Refs: 8053	Poor growth response was obtained inrabbits fed diets containing *Pediococcus*. The apparent nutrient digestibility and the ileal morphology were unaffected. Rabbits fed diets containing *Pediococcus acidilactis* showed a reduction inthe total volatile fatty acid (VFA) concentration. Rabbits fed *Pediococcus* showed higher TBC (total bacterial count) and caecal *Lactobacillus* but also the lowest coliform counts.
Aspergillus spp.	**1** Refs: 9506	*Aspergillus* is supplemented in rabbit diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Candida spp.	**1** Refs: 9506	*Candida* is supplemented in rabbit diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Lactococcus spp.	**1** Refs:	*L. lactis* fortified diet was effective at decreasing pathogenic bacteria colonisation and translocation in a long-term neonatal model. The addition of *L. lactis* to the diet resulted in appropriate growth without colonisation or

	2059	translocation of the probiotic outside the GI tract.
Streptomyces spp.	**1** Refs: 9506	*Streptomyces* is supplemented in rabbit diets as mixtures with other probiotics. Therefore, a specific mode of action could not be attributed to them.
Mucor spp.	**1** Refs: 9506	*Mucor* is supplemented in rabbit diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.6.2 Rabbits – prebiotics

Feed supplementation with mannan oligosaccharides (MOS) and arabinoxylans oligosaccharides (AOS) together with two probiotic bacteria (*Pediococcus acidilactis, Bacillus cereus*) resulted in an increase in microvilli high as well as a reduction of caecal coliform counts (Table 47). Additionally, an increase in the caecal volatile fatty acid concentration was obtained in the MOS supplemented diet. In this paper, MOS supplementation of feed improved the gut health of rabbits.

Table 47: Mode of action of different prebiotics in rabbits

Rabbits - Prebiotic	Number of articles	Mode of action
Mannan oligosaccharide (MOS)	**1** Refs: 8053	Inclusion of prebiotics (MOS) in rations for growing rabbits indicated improved growth and improved gut morphology (villus length was increased). Rabbits fed diets containing MOS showed the highest total VFA concentrationsas well as reduced caecal *Lactobacillus* counts. MOS is able to bind to mannose-specific lectin of gram-negative pathogens that express Type 1-fimbriae such as *Salmonella* and *E. coli*, resulting in their excretion from the intestine.
Arabinoxylan oligosaccharides (AXOS)	**1** Refs: 8053	Inclusion of prebiotics (AX) in rations for growing rabbits showed improved growth and improved gut morphology (villus length was increased).

3.4.6.3 Rabbits – plant extracts

Chicoria intybus was examined in young rabbits, resulting in a significant reduction of NH_3 and a significant increase of VFA in the intestinal content; this indicates a higher fermentation degree in gut microbiota. A significant increase in the appendix and follicles size was also observed, indicating direct stimulation of the immune system associated with gut mucosa. The level of neutral detergent-soluble fibre in the feed was examined in another

paper. In this work, the increase of neutral detergent-soluble fibre reduced mortality, improved the intestinal mucosa morphology (increased microvilli and reduction of crypt depth), improved immune response, and reduced the amount of *Clostridium perfringens*.

In study 10571, the observed effects can be explained by the supplementation with the bacterium included in the tested product (*Enterococcus faecium* CCM7420) but not by supplementation with *Eleutherococcus senticosus* plant extract.

Table 48: Mode of action of different plant extracts in rabbits

Rabbits – Plant extract	Number of articles	Mode of action
Soybean derivatives	**1** Refs: 3727	Oat hulls and a soybean concentrate were added to the diet in order to obtain a lower soluble fibre diet. Mucosal atrophy and decreased enterocyte functionality were observed in the animals fed with a low soluble fibre diet; these might cause enhanced permeability of the mucosa to pathogens. Lower levels of soluble fibre (P = 0.074) tended to increase cellular immune response (CD8+ lymphocytes). A decrease in the level of soluble fibre also tended to increase the frequency of detection of *Campylobacter spp*.
Beta vulgaris	**1** Refs: 3727	Beet and apple pulpwere added to the diet in order to obtain a greater soluble fibre diet. The mortality rates decreased with the increased presence of soluble fibre in the diet. An increase in dietary soluble fibre increased villous height of the jejunal mucosa in weaned rabbits. In addition, rabbits showed a reduction in their crypt depth and an increase in the villous height/crypt depth ratio. Rabbits fed with the highest levels of soluble fibre tended to show reduction in the ileal frequency of detection compatible with *Clostridium perfringens*. Soluble fibre has a protective effect on the mucosa, improving immune response.
Chicory	**1** Refs: 1567	Dietary administration of fresh chicory to young rabbits prior to weaning improved the biochemical traits of the caecum content: the lower ammonia (NH_3) and pH values and the higher VFA value indicated improved balance fermentation of gut microflora. Significant increase in the size of the appendix and its follicles is associated with fewer follicles. The chicory-fed rabbits had increased solid food ingestion, as well as an improved daily gain, but the slaughtering weight and feed efficiency were almost the same.
Malus domestica	**1** Refs: 3727	Beet and apple pulp were added to the diet in order to obtain a greater soluble fibre diet. The mortality rates decreased with the increased presence of soluble fibre in the diet. An increase in dietary soluble fibre increased villous height of the jejunal mucosa in weaned rabbits. In addition, rabbits showed a reduction in crypt depth and an increase in the villous height/crypt depth ratio. Rabbits fed with the highest levels of soluble fibre tended to show a reduction in the ileal frequency of detection that is compatible with *Clostridium perfringens*. Soluble fibre has a protective effect on the mucosa, which improves immune response.
Avena sativa	**1** Refs: 3727	Oat hulls and a soybean concentrate were added to the diet in order to obtain a lower soluble fibre diet. Mucosal atrophy and decreased enterocyte functionality were observed in animals fed with a low soluble fibre diet, and these might cause an enhanced permeability of

		the mucosa to pathogens. Lower levels of soluble fibre (P=0.074) tended to increase cellular immune response (CD8+ lymphocytes). Decrease in the level of soluble fibre also tended to increase the frequency of detection of *Campylobacter spp.*.
Eleutherococcus senticosus	**1** Refs: 10571	Dietary supplementation with *E. senticosus* extract could improve growth performance, stimulate digestive immunity, and increase the host's defence capacities by increasing phagocytic activity and reduction/competition with potential pathogens, without induction of oxidative stress in rabbits.

3.4.6.4 Rabbits – animal by-products

In the present literature review, there were no studies evaluating animal by-products in rabbits.

3.4.6.5 Rabbits – other substances

The effects of feed supplementation, with both glutamine and a combination of glutamine-arginine, were examined with regard to intestinal health in twenty-five-day-old weaned rabbits. Rabbits fed glutamine supplemented diet showed a decreased in lesions caused by *Eimeria* spp. but showed no significant reduction inoxidative stress (MPO or PPARg activity). *Clostridium* spp. was reduced at ileal level along withother significant effects on the microbiota of caecum. No significant effects were observed on either mucosal histology orsystemic immune response.

Caproic and caprylic acid, two medium-chain organic acids with antibacterial effects, were examined in one paper. In this trial, conducted on rabbits experimentally infected with *Escherichia coli*, a highly significant reduction inmortality was observed. Additionally, a significant increase in microvilli high was obtained through supplemented diets. The increase in the area of follicles of Peyer's patches, produced by *Escherichia coli* experimental infection, was reduced in the supplemented feeds.

Table 49: Mode of action of other substances / agents in rabbits

Rabbits - Other	Number of articles	Mode of action
Organic acids derivatives / Fatty acids	**1** Refs: 9224	No significant differences were reported regarding performance. The replacement of Zn bacitracin with a blend of medium-chain organic acids (caproic and caprylic acids) in the presence of colistin increased jejunal villus height, reduced average follicle area in the caudal ileal Peyer's patch, and did not increase mortality rate.

Amino acids and derivatives	**1** Refs: 1641	1% L-Gln supplementation to postweaned rabbit diets decreased fattening mortality, modified the intestinal microbiota (frequency of *Helicobacter spp*. was decreased in the ileum and cecum and *Clostridium spp.* were reduced in the ileum), although no beneficial effects were observed on mucosal integrity or inflammatory and systemic immune response. Diets containing a combination of 1% Gln and 0.5% Arg were of little additional benefit.

3.4.7. Equine

3.4.7.1 Equine – probiotic

The systematic review reported eight studies of probiotics in equines (Table 50), three studies for *Lactobacillus spp.*, another two for *Enterococcus spp.* and *Saccharomyces spp.* and one for *Bifidobacterium spp.* Due to the reduced number of articles in each group, a summary of the mode of action for each group cannot be described in this report.

Table 50: Mode of action of different probiotics in equine

Equine – Probiotic	Number of articles	Mode of action
Lactobacillus spp.	**3** Refs: 12283 5936 11100	Adequate colonisation of *Lactobacillus rhamnosus GG* (LGG) occurred in foals, but intestinal colonisation in adults was only achieved with a prohibitively high dose. Supplementing equine diets with *Lactobacillus acidophilus* had limited effects on nutrient digestibility or on reducing the risk of acidosis associated with feeding high-starch concentrates to horses.
Enterococcus spp.	**2** Refs: 5936 11100	*Enterococcus* is supplemented in equine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Saccharomyces spp.	**2** Refs: 1149 5936	*S. boulardii* survived in horses with gastrointestinal illness (antimicrobial-associated enterocolitis), but no statistically significant differences were found after several days; there was no return to normal faecal consistency, resolution of watery diarrhoea, return to normal heart rate, respiratory rate, or temperature. Resolution of leucopaenia, attitude improvement, appetite improvement, and survival at discharge did not occur.
Bifidobacterium spp.	**1** Refs: 11100	*Bifidobacterium* is supplemented in equine diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.7.2 Equine – prebiotic

The systematic review reported four studies of prebiotics in equines (Table 51), two studies for FOS, and one each for GOS and AOS. Due to the reduced number of articles in each group, a summary of the mode of action for each group cannot be described in this report.

Table 51: Mode of action of different prebiotics in equine

Equine - Prebiotic	Number of articles	Mode of action
Fructooligosaccharide (FOS)	**2** Refs: 11885 9036	Short chain FOS (scFOS) could lead to a beneficial increase in VFA production, such as butyrate, in the equine hindgut. Therefore, scFOS supplementation could be effective in reducing disruption of the microbial populations in the equine hindgut under stressful situations such as acute starch overload. FOS dose-dependently stimulated the LPS induced inflammatory response in equine PBMCs.
Galactooligosaccharides (GOS)	**1** Refs: 11885	GOS dose-dependently stimulated the LPS induced inflammatory response in equine PBMCs.
Acidic oligosaccharides (AOS)	**1** Refs: 11885	AOS is supplemented in equine diets as a mixture with other prebiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.7.3 Equine – plant extract

The systematic review reported only one study of plant extracts (*Plantago* derivatives) in equines (Table 52). Due to the reduced number of articles, a summary of the mode of action in each group cannot be described in this report.

Table 52: Mode of action of different plant extracts in equine

Equine – Plant extract	Number of articles	Mode of action
Plantago derivatives	**1** Refs: 5936	Psyllium is supplemented in equine diets as a mixture with other probiotics and prebiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.7.4 Equine – animal by-products

In the present literature research, no studies were found that evaluated animal by-products in equines.

3.4.7.5 Equine – other substances

The systematic review reported two studies of other substances / agents (minerals) in equines (Table 53). Due to the reduced number of articles, a summary of the mode of action in each group cannot be described in this report.

Table 53: Mode of action of other substances / agents in equine

Equine – Other	Number of articles	Mode of action
Minerals	**2** Refs: 5666 1262	**BARODON (Anionic Alkali Mineral Complex**) is a potential immunostimulant and an alternative to antimicrobial feed additives for improving equine immune responses. Its use often results in the improved capability of horses to endure an attack of infectious respiratory diseases. Treated groups showed higher proportions of cells expressing major histocompatibility complex class II and CD2, $CD4^+$, $CD4^+$ $CD25^+$, $CD8^+$, and $CD8^+$ $CD25^+$ T-lymphocytes, dendritic cells, surface immunoglobulin M^+ B-lymphocytes in peripheral blood, and enhanced cell proliferative responses with phytohemagglutinin and increased phagocytic activity against *Streptococcus equi* and *Staphylococcus aureus* strains. The proposed action mechanism of nonspecific immunomodulatory preparations is macrophage activation, and the subsequent release of cytokines might enhance the immune response. **Selenium**: Se status (either high or low) did not affect the ability of the horses to mount an immune response to a novel antigen or to a previously administered antigen as measured by antigen-specific antibody production. However, cell mediated immunity, evaluated using in vitro mitogen stimulation, appeared to be suppressed in horses with low Se status, as measured by relative mRNA expression of cytokines in PBMC. In addition, *in vivo* relative cytokine gene expression was also affected by Se status.

3.4.8. Pets

With regard to pets, the most common additives studied, in terms of their effects on immunity, were probiotics and prebiotics.

3.4.8.1 Pets – probiotics

Of the probiotics (21 studies), most were centred on *Lactobacillus spp.* (13 dogs, one in cats), *Enterococcus spp.* (8 in dogs, two in cats), and *Bifidobacterium spp.* (six in dogs) (Table 54). *Lactobacillus* transitorily colonises the gastrointestinal tract of dogs, reducing the concentration of other bacteria and ammonia and increasing lactic acid. One specific *Lactobacillus* affected the total protein and lipid concentrations in the serum of dogs while

reducing glucose concentration (potentially due to higher absorption of some nutrients, although serum protein and lipid concentrations do not necessarily reflect nutrient absorption). Several studies found increased IgA and a better cytokine profile. One study with recombinant *L. Casei* (expressing canine granulocyte macrophage colony stimulating factor (cGM-CSF) protein) resulted in a higher IgG response in dogs vaccinated with CCV (canine coronavirus vaccine). No clear effects were seen in cats; in these cases, *Lactobacillus* was administered as a mixture.

The use of *Enterococcus* in dogs (eight studies) decreased other *Enterococcus* (likely due to competitive interactions), increased IgA and TNF, and resulted in a stronger vaccination response (more IgG and IgA) to vaccination with CDV (Canine Distemper Virus). In cats (two studies), this resulted in a more stable microbiome and decreased morbility associated with feline herpes virus-1 in some cats. *Bifidobacteria* in dogs increased bifidobacteria and reduced bacteroides counts in faeces.

Table 54: Mode of action of different probiotics in pets

Pets - Probiotic	Number of articles	Mode of action
Lactobacillus spp.	**13** Refs: 3462 7899 3402 10086 824 1912 10960 6783 2480 12282 9019 315 10087	**Cats** (1): *Lactobacillus* is supplemented in cat diets as a mixture with other probiotics and prebiotics. Therefore, a specific mode of action could not be attributed to it. **Dogs** (13): *Lactobacillus* strains are able to survive gastrointestinal passage and transitorily colonise the dog intestine. It reduces the counts of C. *perfringens, E. coli, Staphylococcus,* and *Enterococcus. Lactobacillus animalis* also reduced ammonia and increased lactic acid concentration. The addition of *L. fermentum* AD1 to the diet significantly increased the total protein and lipid and significantly decreased the concentration of glucose in the bloodstream of dogs. Supplementation with *Lactobacillus* increases the concentration of IgA. A cocktail of the three *Lactobacillus spp*. (NCC2628, NCC2766, NCC2767) exerted beneficial effects on cytokine expression primarily through regulatory T-cells (up-regulation predominantly of IL-10 and, to a lesser extent, of TGF-1, leading to an overall decrease in the pro-inflammatory/regulatory cytokines ratio). *L. casei* expressing cGM-CSF protein stimulates production of monocytes and CCV-specific IgG in CCV vaccinated dogs.
Enterococcus spp.	**9** Refs: 3462 10169 731 10578 3402 6817 10087 10170	**Cats** (2): Cats fed *Enterococcus faecium* SF68 had a more stable microbiome. The administration of the probiotic decreased morbidity associated with chronic feline herpes virus (FHV)-1 infection in some cats. There was no detectable enhancement of FHV-1 serum IgG antibody responses or lymphocyte responses to concanavalin A or FHV-1 antigens in SF68-supplemented cats. **Dogs** (8): A decrease of total *Enterococci* by supplementation of dog diets with *Enterococcus faecium* strain EE3 is likely due to competitive interactions (e.g. by lactic acid) of the EE3 strain with other enterococci. Local effects of the EE3 strain application included an increase in LAB and a significant decrease of *Pseudomonas*-like bacteria and *Staphylococci*.

	5964	The total IgA in the faecal contents and plasma tended to be increased in *Enterococcus* fed animals. *Enterococcus faecium* increased TNF levels. There was a greater proportion of mature B-cells (CD21/MHCII) in puppies fed SF68 at week 31 and 44. The surface expression of the MHC-II molecule in the monocyte population was higher in puppies fed SF68 at week 44. The response to CDV vaccination was stronger in puppies supplemented with SF68, as evidenced by increased amounts of both CDV-specific IgG and IgA in the test group.
Bifidobacterium spp.	**6** Refs: 7900 7899 315 10087 5300 3462	**Dogs** (6): Animals fed diets supplemented with *Bifidobacterium* showed an increase in *Bifidobacterium spp*. as well as a transient decrease in *Bacteroides spp*. Therefore, supplementation with a canine-derived probiotics comprised of *Bifidobacterium* can increase the physical welfare of dogs.
Bacillus spp.	**2** Refs: 3462 3402	*Bacillus* is supplemented in pet diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Streptococcus spp.	**2** Refs: 315 3462	*Streptococcus* is supplemented in pet diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Candida spp.	**1** Refs: 9019	*Candida* is supplemented in pet diets as a mixture with other probiotics. Therefore, a specific mode of action could not be attributed to it.
Weissella spp.	**1** Refs: 6783	*Weissella* survived (LAB10) in the upper gastrointestinal tract and modified the dominant pre-existing indigenous jejunal LAB microbiota of the dogs. When the LAB supplementation was ceased, Denaturing Gradient Gel Electrophoresis (DGGE) analysis of jejunal chyme showed that all the fed LAB strains were undetectable after seven days.

3.4.8.2 Pets – prebiotic

The most commonly studied prebiotics were fructooligosaccharades (FOS, nine studies in dogs, two in cats), mannanoligosaccharides (MOS, four studies in dogs), inulin (source of FOS, four studies in dogs) and yeast wall (source of MOS among other substances, three studies in dogs) (Table 55). Regarding FOS, this altered faecal bacterial composition in dogs, reduced putrefactive compounds, and decreased the acetic + butyric: propionic ratio. Their supplementation increased IgM in colostrum and milk in dogs and attenuated the response to infection (milder decreases in food intake, body weight, and temperature) including lower enterocyte sloughing. In cats, they increased *Bifidobacteria* and tended to reduce the percentage of blood lymphocytes. The inclusion of MOS tended to increase *Lactobacillus* and decrease total anaerobes in the faeces of dogs, as well as increasing serum IgA and % of

blood lymphocytes. There was faster remission of diarrhoea in dogs infected with EPEC. One negative consequence was the tendency to reduce the apparent digestibility of dry and organic matter. Inulin in dogs resulted in increased faeces production, increased *Lactobacillus*, and lower crude protein apparent in faecal digestibility. In dogs, the yeast cell wall increased *Bifidobacterium*, reduced *E. coli*, reduced the percentage of white blood cells in blood, and tended to increase IgA in serum and ileum.

Table 55: Mode of action of different prebiotics in pets

Pets - Prebiotic	Number of articles	Mode of action
Fructooligosaccharide (FOS)	**10** Refs: 246 11084 823 3402 11082 13139 5159 7218 11828 3462	**Cats** (2): Cats fed scFOS-supplemented diets had increased faecal *Bifidobacterium spp*. populations. Lymphocyte concentrations (expressed as a percentage) were generally reduced for cats fed the scFOS treatment. **Dogs** (9): Fructans can influence the bacterial composition of the gut microbiota in healthy dogs. Faecal *S. lutetiensis* isolates represent the *Streptococcal* group that appeared to be involved in one of the most pronounced population changes observed after fructan administration. Furthermore, fructooligosaccharides decrease concentrations of putrefactive compounds measured in faeces, improving gut health. FOS increased propionic acid and reduced the acetic + n-butyric to propionic acid ratio. FOS supplementation also decreased the concentrations of selected protein catabolites formed in the large bowel. The supplementation of feed by scFOS is concomitant of higher IgM in colostrum and milk must thus afford increased protection of the neonate's mucosae. FOS attenuated the decreased food intake, body weight changes, and increased body temperature caused by infection. FOS decreased enterocyte sloughing, suggesting a protective role during infection. Infected puppies that consumed scFOS tended to have a greater villus width.
Mannan oligosaccharide (MOS)	**4** Refs: 11084 3402 11082 3797	**Dogs** (4): Mannan oligosaccharides tend to increase *Lactobacillus* numbers and decrease total aerobe concentration. By producing lactate and bacterocins, lactate-producing bacteria reduce colonic pH and decrease pathogen populations). Lymphocytes (% of total white blood cells) and serum IgA were increased in dogs supplemented with MOS. Dogs supplemented with MOS tended to have reduced ileal dry matter (DM) and organic matter (OM) digestibilities when compared with the dogs in the control group. Dogs infected with enteropathogenic *E. coli* (EPEC), receiving MOS, demonstrated faster remission of diarrhoea. MOS binds to the type-1 mannose site in the bacterium, preventing the latter from binding to the glycoproteins of intestinal cells.
Inulin	**4** Refs: 823 693 11919 11828	**Dogs** (4): Faeces production was increased by inulin. It decreased the apparent digestibility coefficient of crude protein due to increased faecal moisture content and increased *Lactobacillus*.
Yeast cell wall (YCW)	**3** Refs:	**Dogs** (3): YCW also increased *Bifidobacterium*. It also decreased *E. Coli,* likely due to the ability of the mannose moiety to bind to type-1 fimbrae as they are expressed by *E. coli* and *Salmonella spp*. This binding prevents

	7219 693 7218	these bacteria from adhering to the intestinal wall and colonising the intestine. White blood cell and eosinophil counts tended to decrease quadratically with YCW supplementation, whereas monocyte counts decreased in a linear fashion. Serum immunoglobulin A (IgA) concentrations tended to respond cubically to YCW, and ileal IgA tended to respond quadratically.
Galactooligosaccharides (GOS)	**2** Refs: 7900 5159	**Dogs** (1): *Bifidobacterium bifidum* 02 450B growth was stimulated by GOS **Cats** (1): GOS-supplemented diets increased the counts of faecal *Bifidobacterium spp*.
Polydextrose	**1** Refs: 694	**Dogs** (1): Polydextrose increased concentrations of faecal SCFA, primarily acetate and propionate, by decreasing faecal pH, without affecting food intake or faecal output. Polydextrose also decreased some protein catabolites, in particular faecal indole concentrations. Faecal *C. perfringens* concentrations were decreased, and *Bifidobacteria* increased, by the inclusion of polydextrose in the diet.
Arabinogalactan	**1** Refs: 3462	Arabinogalactan is supplemented in pet diets as a mixture with other probiotics and prebiotics. Therefore, a specific mode of action could not be attributed to it.

3.4.8.3 Pets – plant extracts

There were only a few studies on plant extracts (beet pulp, chicory, and *Plantago* derivatives; Table 56). All of these explored prebiotic fibre (beet pulp containing a mix of fermentable and non- fermentable fibres and chicory provides inulin which, in turn, provides FOS-, and *Plantago*, which provides soluble fibres). Due to the reduced number of articles, a summary of the mode of action in each group cannot be described in this report.

Table 56: Mode of action of different plant extracts in pets

Pets – Plant extract	Number of articles	Mode of action
Beta vulgaris	**2** Refs: 823 7218	**Dogs** (2): Beet pulp can increase *Bifidobacteria* in the canine intestine. The high concentration of SCFA in the beet pulp treatment is likely caused by the fact that beet pulp contains a blend of several fermentable substrates, all with different fermentation rates.
Chicory	**1** Refs: 823	**Dogs** (1): Mode of action is not described in this study.
Plantago derivatives	**1** Refs: 823	**Dogs** (1): Mode of action is not described in this study.
Pisum sativum	**1** Refs: 823	**Dogs** (1): Mode of action is not described in this study.

Larix	**1** Refs: 3855	**Dogs** (1): Dogs whose diets were supplemented with larix showed increased concentration of total aerobic faecal bacteria, higher concentrations of faecal *Lactobacillus* and *Bifidobacteria*, and lower concentration of faecal *Clostridium perfringens*. Specific forms and doses of arabinogalactan (AG) increased white blood cell, neutrophil, and eosinophil concentrations.
Cyamopsis derivatives	**1** Refs: 823	**Dogs** (1): Mode of action is not described in this study.

3.4.8.4 Pets – animal by-products

The systematic review reported only one study of animal by-products (lactoferrin) in pets (Table 57). Lactoferrin did not indicate any clear effects on immunity.

Table 57: Mode of action of different animal by-products in pets

Pets – Animal by-product	Number of articles	Mode of action
Lactoferrin	**1** Refs: 4103	**Dogs** (1): Lactoferrin did not alter the intestinal morphology and had a minor impact on colonisation with lymphocytes and plasma cells. $CD8^+$ lymphocytes were increased in the epithelium of the colon of the lactoferrin groups.

3.4.8.5 Pets – other substances

The systematic review reported three studies of other substances / agents in pets (Table 58). Due to the reduced number of articles in each group, a summary of the mode of action for each cannot be described in this report.

Table 58: Mode of action of other substances / agents in pets

Pets - Other	Number of articles	Mode of action
Others	**2** Refs: 10170 1815	**Dogs** (1): **Toll-like receptor (TLR) ligands** might induce a greater anti-inflammatory gene expression profile in vivo in dogs. **Cats** (1): Domestic cats can readily absorb **β-carotene** across the intestinal mucosa. β-carotene is also is taken up by peripheral blood leukocytes and is distributed into subcellular organelles, notably the mitochondria. β-carotene may play an important role in maintaining the structural and functional integrity of leukocytes.
Vitamins	**1** Refs: 3402	Vitamin B is supplemented in dog diets as a mixture with other probiotics and prebiotics. Therefore, a specific mode of action could not be attributed to it.

3.5. Interactions of the substances / agents identified with other dietary compounds

Seventy-one interactions have been detected between the substances / agents and other dietary compounds.

The main primary interactions studied were those between two or more immunomodulatory substances / agents added to the diet. The aim of studying these was to assess whether or not the effects induced by these have a synergistic or additive effect.

Twenty-four, 25, and 18 studies regarding interactions were explored in porcine, poultry and fish populations respectively. Only four studies were found for ruminants. In rabbits, equines, and pets, studies with interactions were not found within this systematic review. More information on these interactions can be found in the tables for porcine (Table 59), poultry (Table 60), bovine (Table 61), ovine and caprine (Table 62), and fish populations (Table 63).

3.5.1. Porcine

Table 59: Interaction of substances / agents identified with other dietary compounds in porcine

Refid	Interaction	Description of the interaction	Assessment	Type of interaction
945	*E. faecium* x inulin	A combination of *E. faecium* and inulin improves the survival of the probiotic strain through the upper intestinal tract and allocates the symbiotic effect.	Qualitative	Synergistic
1235	*E. faecium* SF68 x ZnO	An *E. faecium* SF68/ZnO interaction was observed for lactic acid bacteria in the small intestine tissue.	Quantitative	Other
1301	BioPlus x FOS	Synbiotic increased duodenal micro-villous height at the 14th day after weaning.	Quantitative	Other
1389	Glutamine x glutamate	The supplementation of glutamine and glutamine plus glutamate (AminoGut) in pre- and post-weaning diets improved feed conversion in the first three week post-weaning when compared to control treatment.	Quantitative	Additive
1570	BioMos x Bioplex Zn	Crypt depths in the jejunum were lower in animals fed the combination of additives (BioMos+Bioplex), and there was also an increase in villus:crypt ratio.	Qualitative	Additive
1873	Antibiotic x multimicrobe probiotic	The addition of antibiotics to probiotic diets improved the performance of weaning piglets but did not indicate any interaction with probiotics.	Qualitative	Other
1874	Multimicrobe probiotic x antibiotic	The main effects of probiotic products (liquid or solid feed), antibiotics (colistin or lincomycin), and their interaction were determined but this interaction (probiotic x antibiotic) was not statistically significant.	Quantitative	Other
1916	Flax-seed oil x	Combined treatment down-regulates IL-1a and IL-8	Qualitative	Other

	Lactobacillus plantarum – Biocenol™ LP96	gene expression, up-regulates IFN-c, and tends to regulate inflammation induced by ETEC through cytokine IL-10.		
2001	*Bifidobacterium lactis* Bb12 x *Lactobacillus rhamnosus* LGG	*B. lactis* Bb12 and *L. rhamnosus* LGG in combination revealed an improved ability to inhibit adhesion of all pathogens tested in pig intestinal mucus when compared to probiotic strains.	Quantitative	Synergistic
2496	*B. subtilis* RJGP16 x *L. salivarius* B1	This study demonstrates that dietary co-administration of the *B. subtilis* RJGP16 and L. salivarius B1 is more effective at stimulating local mucosal immunity than a single probiotic would be	Quantitative	Synergistic
2859	β-glucan x vitamin C	Cortisol concentrations indicated an interaction between β-glucan and vitamin C. There was a tendency for a vitamin C and β-glucan interaction in plasma TNF-α concentration following LPS administration. There was trend for a time × treatment interaction for the ADG. Plasma cortisol profiles indicated a trend for an effect of vitamin C and a trend for a β-glucan and vitamin C interaction in response to the challenge.	Quantitative	Synergistic
3937	Lactulose x *Lactobacillus*	The synbiotic combination combines all the aforementioned positive effects, although we were not able to demonstrate a specific growth of *L. plantarum* promoted by lactulose, suggesting that this combination is acting as a complementary symbiotic and not as a synergic one.	Quantitative - Qualitative	Additive
4555	SDPP x Yeast Protein	The increasing serum levels of IgM and IgA may be associated with improved intestinal immunity resulting from the synergistical effect of combining SDPP with yeast-derived protein (YP).	Quantitative	Synergistic
4709	Quillaja saponin x curcumin	No interactions between Quillaja saponin and curcumin supplementation were observed for any of the measurements.	Quantitative	Other
4972	LPS X Gly-Gln	There was an LPS challenge × diet GlyGln interaction for ADFI, but it was difficult to ascertain whether or not Gly-Gln increased ADFI. A trend for an LPS challenge × diet Gly-Gln interaction was observed for ADG. There was no interaction of LPS challenge × Gly-Gln addition for IL-1β concentration. There were no interactions between LPS challenge and Gly-Gln for IL-2 and sIL-2R.	Quantitative	Other
5732	potato starch x probiotic	The second-highest ADG was seen with the probiotic alone, and there appeared to be a biological interaction between the potato starch and the probiotics.	Quantitative	Other
6009	fat source x vitamin E	No significant interactions between fat source and vitamin E were observed.	Quantitative	Other
6082	Organic acids x nucleotides	Dietary supplementation with organic acids and nucleotides has a synergetic effect on the Peyer's patches and mesenteric lymph node lymphocyte proliferation.	Quantitative	Synergistic
6186	Seaweed extract x fish oil	No significant interaction was observed between maternal SWE and FO supplementation on PW pig performance. In the caecum, there was an interaction between maternal SWE and FO supplementation on E. coli populations and *Lactobacillus*:*E. coli* ratio in pigs 9 d PW. Piglets weaned from SWE-supplemented sows had a reduced *E. coli* population and higher	Quantitative	Other

		Lactobacillus:E. coli ratio in the caecum when compared with pigs weaned from sows fed the basal diet. However, when the combination of SWE and FO was offered to sows, no effects were detected on *E. coli* numbers or*Lactobacillus*:*E. coli* ratio compared with FO-only diets. In the colon, there was a significant interaction between maternal SWE and FO supplementation on the molar proportions of valeric acid, isovaleric acid, isobutyric acid, and total branched-chain fatty acid. FO supplementation induced an increase in molar proportions of valeric acid, isovaleric acid, isobutyric acid and branched-chain fatty acids compared with the basal diet; however, there was no effect on molar proportions of valeric acid, isovaleric acid, isobutyric acid, or branched-chain fatty acids with the combination treatment.		
6429	LPS x Diet	There was an LPS challenge x diet interaction observed for jejunal HSP70. There was an LPS challenge x diet interaction observed for jejunal TLR4 and ileal IL-1 receptor-associated kinase 1 (IRAK1). An LPS challenge x diet interaction was observed for jejunal NOD2 and Receptor-interacting serine / threonine-protein kinase 2 (RIPK2) and ileal NOD2.	Quantitative	Synergistic
6718	Inulin x Probiotics	Inulin applied alone seemed to depress the activity of enterococci in the colon. The combination of inulin and the probiotic formulation in the present study prevented this reduction, resulting in a significantly increased enterococci number in the synbiotic group compared to the control and inulin group and a non-synergistic interaction of the experimental factors. In the present study, in digesta obtained from colon, enterobacterial numbers were numerically lower in the prebiotic and probiotic groups than in the synbiotic group. Furthermore, the determination of the *Lactobacilli*:*Enterobacteria* ratio revealed this significant interaction between the experimental factors not only in the colon but also in jejunum and ileum. In these three observations, the ratio was more favourable in inulin and probiotic groups whereas for synbiotics the values remained unchanged compared with the control.	Quantitative	Antagonistic - Synergistic
11515	Trp x F4R+	Interactions between Trp and F4R presence were observed for REG3G, SFTPD, complement factor B (CFB), LBP and TLR4. For these genes, a linear effect of F4R presence in the low Trp group was identified.	Quantitative	Other
11516	Feed x Probiotic level	An interaction between the inclusion of bFOSs and the dose of *B. animalis* in the diet was seen for the expression of TLR2-encoding gene in the ileocaecal MLNs.	Quantitative	Additive
12118	Laminarin x Fucoidan	There was an interaction between LAM and FUC supplementation on duodenal villus height and the villus height to crypt depth ratio.	Quantitative	Synergistic

3.5.2. Poultry

Table 60: Interaction of substances / agents identified with other dietary compounds in poultry

Refid	Interaction	Description of the interaction	Assessment	Type of interaction
1164	Essential oil of oregano (HMO) x hop extract (HMH)	Non-synergistic or additive mechanisms in terms of a combined inclusion (HMOH).	Quantitative	Other
1165	Diet type (corn or wheat) x EOM (essential oil mixture)	The obtained increases in female BW, with respect to EOM supplementation to the corn-based diet, were inferior to those in the wheat-based diet. The weight of the spleen yielded variable responses to different treatments, with significant interactions between diet and EOM.	Qualitative	Additive
1408	Commercial synbiotic (*E. Facium* + FOS) x Commercial blend of organic acids and their salts	Commercial synbiotic and commercial blend of organic acids and their saltsdid not cause higher jejunal villus height.	Qualitative	Other
1742	*Bacillus* x *Saccharomyces*	In trial 3, *Bacillus*+*Saccharomyces* fermented feed increased BW and weight gain (WG) at 21- and 39-d-old	Quantitative	Other
2212	Organic acid x LABYuc-Probiotic x peas	There was an interaction between organic acid and probiotic supplementation for liver weight; organic acid supplementation increased liver weight in chickens fed diets lacking probiotics but decreased liver weight in chickens fed diets including probiotics. The interaction Pea X organic acid (OA) X Probiotic was significant, as the total SCFA concentration was highest in birds fed the control diet without OA and with probiotics (517 mmoles/g), followed by the group fed the pea diet with OA and with probiotics (507 mmoles/g).	Quantitative	Other
2909	Phytase (Phy)x Commercial mixture of organic acids	Broilers fed phy + commercial mixture of organic acids had higher levels of IgG in the primary immune response as well as higher total Ig and IgG in the secondary response compared with all other groups, suggesting an additive effect of phytase and organic acid on the immune response to SRBC.	Quantitative	Additive
3433	Clove bud x n-6 / n-3 Fatty Acids ratio	The interaction between different ratios of n-6 to n-3 and clove bud was significant regarding egg shell strength. The interaction between experimental factors showed that feeding the highest level of clove bud significantly improved FCR in both the high and low ratio diets. There were positive interaction effects of bioactive compounds of clove bud and n-3 fatty acids on cell proliferation of such peripheral lymphoid organs as the spleen.	Quantitative	Additive
4516	Enery level x feed additive	No interactions were observed for the measured parameters.	Quantitative	Other
4518	Protein level x (*B. subtilis* and *C. Butyricum*	Significant interactions between additives and protein levels were not observed for antibody response to Newcastle disease or performance traits.	Quantitative	Other

	or MOS)			
4938	BP70 (sodium butyrate) x EO (Ginger oil, carvacrol)	In the combination, BP70 and essential oils might exert a synergistic action as there is a significant difference in gross pathological scores between essential oil (EO) and EO+BP70 groups. As BP70 alone did not have any beneficial effects in this study, it can be hypothesised that the essential oils potentiate the effect of sodium butyrate.	Qualitative	Synergistic
5333	AL (*Artemisa* leaves) x BCS (black curcumin seeds)	No interaction of AL and BCS on BW, FCR, and feed intake was significant. Adding both AL and BCS to the diet significantly altered caecal lactobacilli compared with BCS alone and altered coliform compared with the control group and BCS. This indicated an interaction between these products asthe BCS alone had no effect on caecal bacterial populations.	Quantitative	Other
5360	Arg x Lys	No interactions occurred between dietary Lys and Arg.	Qualitative	Other
6267	APS (*Astragalus* polysaccharides) x Probiotics (*Lactobacillus* and *B.Cereus*)	There were significant increases in the thymus, bursa of Fabricius, or spleen relative weights in the dual fed treatment compared with the probiotics and APS treatments, with the exception of the thymus.	Quantitative	Synergistic
6408	Phytate x phytase	There was no interaction between phytate and phytase on bird performance, erythrocyte rosette-forming cells (ERFC) and erythrocyte-antibody complement cells (EAC). There was no interaction between phytase and phytate on the percentages of T-lymphocyte subsets and their ratios. For the low-phytate diets, phytase addition did not affect the levels of serum anti-NDV antibodies at d 14, 21, and 28, whereas with the high-phytate diets the anti-NDV antibodies were, on average, increased by 0.59 and 0.91 $\log_2$ at d 21 and 28, respectively. This resulted in a significant interaction of phytate and phytase at d 21 and 28 in anti-NDV antibodies. The interaction between phytate and phytase was not significant for SIgA.	Quantitative	Other
7977	MOS x Enzyme (Allzyme Phytase)	A MOS x Enzyme interaction was observed for perimeter and height of duodenum villi that were larger compared to those of negative control chickens.	Quantitative	Other
8174	Vitamin E x Vitamin C	No interaction between the effects of vitamins E and C, on either humoral or cell-mediated immune responses, were observed in the present study. However, supplementing dietary vitamin E at 125 IU/kg or vitamin C at 200 mg/kg, independently alleviated the negative effects of high environmental temperature on immune response. Dietary vitamin E at 125 IU/kg and vitamin C at 200 mg/kg had an additive effect in reducing the activity of oxidative enzyme LP and increasing the activity of antioxidant enzyme GSHR.	Quantitative	Other
8359	Probiotic mixture x MOS	In the duodenum, the highest VH was obtained in the control group, whereas the lowest VH was observed in the groups fed the in-feed probiotic containing only *Bacillus subtilis*. However, VH in these two treatments were not different from the group fed with the bacterial pool used as a probiotic. Higher CD values were obtained in the duodenum of the birds that did not	Quantitative	Other

		receive growth promoters and those fed the individual *Bacillus subtilis* culture, whereas the lowest values were obtained with the use of the bacterial pool.		
8360	Probiotic mixture x MOS	Significant interaction ($p<0.01$) between the evaluated factors was identified for both VH and CD across the three intestinal portions.	Quantitative	Other
8812	Feed form x DFM (Primalac); *Salmonella* × feed × DFM	In the jejunum, there was a feed form × DFM interaction for GC number, where DFM in mash feed increased the GC number. There was a *Salmonella* × feed × DFM interaction in the jejunum for both GC total area and mean size, where in birds challenged with *Salmonella*, the DFM increased GC total area and mean size for birds fed mash feed but not for those fed crumbled feed.	Other	Other
9055	*Saccharomyces* x Thr	An interaction between both parameters was observed in villus width in the ileum, WBC, and antibody titers against Newcastle disease at 26 days.	Quantitative	Other
9276	Metabolite M x organic acids	The combination of this treatment caused a significant lower average feed consumption than in the positive control group. However, combination treatment had better feed conversion ratio than positive, negative and 0.1% A diets. The combination of 0.1% A+ 0.5% M had no significant differences on digesta pH as compared with positive controls and 0.1% A groups. Higher concentration of total VFA levels and acetic acid were found in 0.5% M and 0.1% A + 0.5% M as compared the remainder of the treatments. There were significant differences between controls and the combination of 0.1% A + 0.5% M groups for valeric acid.	Quantitative	Other
9392	Prebiotic (TechnoMOS)x Food restriction x *Salmonella*	Prebiotics caused an increase at day 15, and feed restriction caused a decrease at day 30 in toe web thickness. Interaction among the factors was significant ($P<0.05$). There were no significant differences among treatments and factor interaction regarding antibody titer responses against SRBC at days 17 and 27, and to Newcastle disease virus (NDV) at day 17. However, there were no significant differences among factor interaction regarding antibody titer response against NDV at day 27. There were no significant differences among treatments and factor interaction regarding relative weight of spleen at days 21 and 42.	Qualitative	Other
10008	MOS x threonine	Mannan oligosaccharides and threonine act synergistically, resulting in improved intestinal environment and recovery after Salmonella inoculation.	Quantitative	Synergistic
12935	β-glucan x *Bacillus subtilis*	The interactive effect between *Bacillus subtilis* and β -glucan was only observed in the yellowness (b* value) of breast meat, which may be induced by the lower abdominal fat content.	Qualitative	Synergistic
13087	Arg x Vitamin E	However, 19 d after vaccination, we identified a significant interaction between Arg and vitamin E (VE); at the lower VE supplementation (VE40 and VE80), birds fed NARG feed had a higher CD4+:CD8+ ratio than birds fed the HARG feed, but birds fed the highest VE supplementation (VE200) had a similar CD4+:CD8+ ratio regardless of the Arg supplementation level.	Quantitative	Other

3.5.3. Bovine

Table 61: Interaction of the substances / agents identified with other dietary compounds in bovine

Refid	Interaction	Description of the interaction	Assessment	Type of interaction
2914	*E. faecium* x *S cerevisiae*	Oral supplementation of *E. faecium* alone had no effect on the mediators of the acute phase response that were measured, whereas feeding *E. faecium* and yeast induced an inflammatory response in feedlot steers fed high-grain diets.	Quantitative	Synergistic
5146	Se x IgG (colostrum)	Se supplementation in colostrum increased IgG amount and Se concentration in the blood plasma of newborn calves.	Quantitative	Additive
10190	Vitamin A x Lactoferrin	There were significant interactions between vitamin A and Lf with respect to crypt cell proliferation, not only in the colon but also in the ileum.	Quantitative	Other

3.5.4. Ovine and caprine

Table 62: Interaction of substances / agents identified with other dietary compounds in ovine and caprine

Refid	Interaction	Description of the interaction	Assessment	Type of interaction
5507	MOS × feeding model	A MOS × feeding model interaction ($P<0.001$) was noted. When introduced to HS diets, this supplement increased erythrocyte count by 4.2% and haematocrit value by 4.7%, whereas its presence in the diets based on hay alone had no effect on these parameters. The MOS additive influenced ($P\leq0.05$) white blood cell counts but its impact varied significantly across treatments.	Quantitative	Synergistic

3.5.5. Fish

Table 63: Interaction of substances / agents identified with other dietary compounds in fish

Refid	Interaction	Description of the interaction	Assessment	Type of interaction
956	*Lactobacillus plantarum* x inulin	No clear effect of the interaction shown.	Qualitative	Less than additive
1577	Chromium carbochelate x *Saccharomyces*	In tilapia supplemented with Cr + *S. cerevisiae* and inoculated with *S. agalactiae*, no potentiating effect	Qualitative	Other

	cerevisiae	from the inflammation occurred.		
1605	*Bacillus subtilis* x Microalgae (*Tetraselmis chuii, Phaeodactylum tricornutum*)	Increase in lamina propria lymphocytes.	Quantitative	Additive
1609	*Bacillus subtilis* x inulin	Humoral and cellular immune parameters.	Quantitative	Additive
1610	Interaction between inulin, *B. subtilis* and microalgae	The inclusion of inulin, *B. subtilis,* and microalgae to the diet provoked minor alterations in the gut (both at the microscopic and microbiota levels), which correlate with slight inflammation mediated by Il-8, CASP-1 and COX-2.	Quantitative	Less than additive
1666	*Enterococcus faecium* SF68 x *Bacillus toyoi*	Ensuring these two probiotics are the dominant microflora in the intestine, prior to pathogen invasion, may be the key to disease prevention.	Quantitative	Less than additive
1800	Arginine x glutamine	The combined effects of arginine and glutamine on NBT provide strong evidence of a possible synergy as the production of oxidative radicals was greater in neutrophils from fish fed the arginine–glutamine diet than those fed diets supplemented with arginine or glutamine alone. The same was true with regard to lysozyme activity.	Quantitative	Additive
3533	*Lactococcus lactis* or *Bacillus circulans* x AXOS	Growth parameters were unaffected by the interaction. The interaction effects between AXOS and candidate probiotics significantly affected the relative number of *Lactobacillus aviarius*, *Cetobacterium somerae,* and *Rhodobacter spp. L. aviarius* significantly increased in fish fed diet 2 (AXOS) or diet 7 (basal feed +*L. lactis spp. lactis* ST G45 + AXOS), in comparison to fish fed probiotic diets (diet 1, 3, 4, 5) and diet 8 (basal feed + *B. circulans ST* M53 + AXOS). *L. aviarius* was not detected in fish fed diet 8. Relative abundances of *C. somerae* were significantly higher in the hindgut of fish fed the control diet than in those fed diet 7 (basal feed + *L. lactis spp.* lactis ST G45 + AXOS). The abundance of *Rhodobacter spp.* significantly increased in fish fed a diet supplemented with *L. lactis spp. lactis* ST G81 + AXOS in comparison to the other treatments, with the exception of fish fed the control diet (diet 1), diet 5 (basal feed + *B. circulans* ST M53) or diet 8 (basal feed + *B. circulans* ST M53 + AXOS).	Qualitative	Other
3620	LAB x peptides	No synergistic or cumulative effects were achieved by combining lactic acid bacteria and immuno-stimulating peptides.	Qualitative	Other
6346	COS x *Bacillus coagulans*	Dietary *B. coagulans* and COS had a synergistic effect on enhancing immunity and disease resistance of koi.	Quantitative	Synergistic
7117	*Entercoccus*	Growth, feed utilisation.	Quantitative	Additive

	faecium x FOS			
8384	Soybean protein level x nucleotide supplementation	Interactions, especially synergistic actions, would exist between soybean protein level and nucleotide supplementation level on immunity and distal-intestinal morphometric, not on growth. In the present study, significant interaction between soybean protein level and nucleotide supplementation level has been found forrespiratory burst activity of macrophages in the head kidneys, fold height, and microvillus height in distal-intestinal of turbot. Therefore, supplying nucleotides to the diet at high soybean protein level would benefit the health of turbot.	Quantitative	Synergistic
8597	*Saccharomyces cerevisiae* extract/CpG ODN x SLICE emamectin benzoate (EMB)	Despite the positive effects on infection of fish fed immunostimulatory feeds, no synergism was observed with follow-up treatment with SLICE®.	Quantitative	Other
9051	*Lactobacillus sakei* 5-4 x microalgae *Navicula spp.*	The combined administration of microalgae *Navicula spp.* and *L. sakei* 5-4 (the SM diet) resulted in stimulation of the humoral immune parameters	Quantitative	Additive
12877	FOS x *B. licheniformis*	After *A. hydrophila* challenge, survival rate was unaffectedby either FOS levels or *B. licheniformis* content, whereas a significant interaction between these two substances was observed, with the highest value observed in fish fed 0.3% FOS and $1x10^7$colony forming units (CFU)/g *B. licheniformis*.	Quantitative	Synergistic
12905	*B. subtilis* x FOS	*B. subtilis* and FOS had a synergistic effect on enhancing immune response and disease resistance of the sea cucumber.	Quantitative	Synergistic
12908	*B. subtilis* X FOS	The cumulative percentage of mortalities was significantly affected by both *B. subtilis* and FOS, and a significant interaction was identified between *B. subtilis* and FOS. The respiratory burst activity of fish was significantly affected by the interaction of dietary *B. subtilis* and FOS. Phagocytic activity, alternative complement pathway (ACP) activity, and lysozyme activity were significantly affected by the interaction of dietary *B. subtilis* and FOS.	Quantitative	Synergistic
13185	*B. subtilis* x FOS	Absence of interactions between FOS and *B. Subtilis*.	Other	Other

3.5.6. Other species (rabbits, equine and pets)

Interactions of the substances / agents identified with other dietary compounds were not found for rabbits, equines, or pets.

3.6. End-points to be requested by main substances and target species

In this section, the most important indicators are listed in order to describe the benefits of the application of substances or agents. The end-points are then classified into three main groups for the majority of the species: local immune response, systemic immune response, and health status; with the exception of fish, the end-points are classified as measured immunological parameters and health status.

Tables for each type of substance and major target species are presented. With the numerical description, a potential proposal of end-points can be assessed.

3.6.1. Porcine

3.6.1.1. Probiotics

The end-points studied in porcine fed with probiotics are counted in Table 64.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (75 articles out of a total of 140), representing 53.6 % of the studies included in this section of the present search. The parameters most frequently studied in systemic immune response were immunoglobulins and cytokines (both were included in24 articles, out of a total of 140), representing a 17.1 % of the studies included in this section of the present search. The parameter most frequently studied in health status was performance (79 articles of a total of 140), representing a 56.4 % of the studies included in this section of present search.

Bacteria evaluated in the different studies include pathogenic bacteria (i.e. *E. coli*), commensal bacteria (i.e. LAB), or probiotic intentionally added to the diet. The immunoglobulins analysed include IgG and IgA. In the case of cytokines, the most frequently studied were Il-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ. The performance parameters evaluated included: body weight, average daily gain, average daily feed intake, and feed conversion ratio.

Table 64: Number of studies for each end-point when probiotics are studied in porcine

PORCINE – PROBIOTIC (n=140)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	31
	Oxidative stress	5
	Cytoquine responses	27
	Immunoglobulins	14
	Phenotypic changes in lymphoid cells	13
	Intestinal microbiota	75
	Metabolomic studies	2
	Others	36
Systemic immune response	Acute phase proteins	6
	Immunoglobulins	24
	Cytokines	24
	Phagocytic activity of lymphoid cells	5
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	7
	Phenotypic changes in lymphoid cells	14
	Weight lymphoid organs	2
	Other	35
Health status	Diarrhoea	27
	Mortality	6
	Morbility	1
	Performance	79
	Carcass traits	3
	Skeleton	0
	Other	11
	Not defined	49
Number of studies with challenge		47

3.6.1.2. Prebiotics

The end-points studied in porcine fed with prebiotics are counted in Table 65.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (22 articles of a total of 57), representing 38.6 % of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was cytokines (15 articles out ofa total of 57), representing 26.3 % of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (24 articles out of a total of 57), representing 42.1% of the studies included in this section of the present search.

Bacteria evaluated in the different studies include pathogenic (i.e. *E. coli*) and commensal bacteria (i.e. LAB). The cytokines analysed include Il-1β, IL-6, IL-10, TNF-α and IFN-γ. The

performance parameters evaluated include: body weight, average daily gain, average daily feed intake, and feed conversion ratio.

Table 65: Number of studies for each end-point when prebiotics are studied in porcine

PORCINE – PREBIOTIC (n= 57)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	14
	Oxidative stress	2
	Cytoquine responses	11
	Immunoglobulins	5
	Phenotypic changes in lymphoid cells	3
	Intestinal microbiota	22
	Metabolomic studies	1
	Others	18
Systemic immune response	Acute phase proteins	4
	Immunoglobulins	13
	Cytokines	15
	Phagocytic activity of lymphoid cells	3
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	5
	Phenotypic changes in lymphoid cells	4
	Weight lymphoid organs	0
	Other	17
Health status	Diarrhoea	5
	Mortality	1
	Morbility	0
	Performance	24
	Carcass traits	0
	Skeleton	0
	Other	3
	Not defined	30
Number of studies with challenge		20

3.6.1.3. Plant extract

The end-points studied in porcine fed with plant extract products are counted in Table 66.

The parameters most frequently studied with regard to local immune response were intestinal microbiota and microvilli structure (18 and 17 articles, respectively out of a total of 48), representing 37.5 and 35.4% of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was immunoglobulins (13 articles out of a total of 48), representing 27.0 % of the total studies included in this section of present search. The parameter most frequently studied in health status was performance (31 articles out of a total of 48), representing 64.6% of the studies included in this section of the present search.

Bacteria evaluated in the different studies include pathogenic (i.e. *E. coli* and *Salmonella*) and commensal bacteria (i.e. LAB). In the case of microvilli structures, the parameters assessed included villus height and crypt depth. The immunoglobulins analysed included IgA, IgG, and IgM. The performance parameters evaluated included: body weight, average daily gain, average daily feed intake, and the feed conversion ratio.

Table 66: Number of studies for each end-point when plant extract products are studied in porcine

PORCINE – PLANT EXTRACT (n = 48)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	17
	Oxidative stress	4
	Cytoquine responses	10
	Immunoglobulins	5
	Phenotypic changes in lymphoid cells	3
	Intestinal microbiota	18
	Metabolomic studies	0
	Others	17
Systemic immune response	Acute phase proteins	5
	Immunoglobulins	13
	Cytokines	11
	Phagocytic activity of lymphoid cells	9
	Cytolytic activity in lymphoid cells	1
	Lymphocyte proliferation activity	7
	Phenotypic changes in lymphoid cells	5
	Weight lymphoid organs	2
	Other	17
Health status	Diarrhoea	8
	Mortality	0
	Morbility	0
	Performance	31
	Carcass traits	0
	Skeleton	0
	Other	6
	Not defined	13
Number of studies with challenge		10

3.6.1.4. Animal by-products

The end-points studied in porcine fed with animal by-products are counted in Table 67.

The parameter most frequently studied with regard to local immune response was microvilli structure (15 articles out of a total of 26), representing 57.7% of the studies included in this section of the present search. The parameters most frequently studied with regard to systemic immune response were cytokines and immunoglobulins (nine and eight articles out

of a total of 26, respectively), representing 34.6 and 30.8% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (18 articles out ofa total of 26), representing 69.2 % of the studies included in this section of the present search.

The microvilli structure analysis includes villus height and crypt depth. The immunoglobulins evaluated include IgA, IgG, and IgM. In the case of cytokines, the most frequently studied were Il-1β, IL-6, IL-10, TNF-α, and IFN-γ. The performance parameters evaluated included: body weight, average daily gain, average daily feed intake, and feed:gain ratio.

Table 67: Number of studies for each end-point when animal by-products are studied in porcine

PORCINE – ANIMAL BY-PRODUCT (n = 26)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	15
	Oxidative stress	1
	Cytoquine responses	7
	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	2
	Intestinal microbiota	7
	Metabolomic studies	0
	Others	5
Systemic immune response	Acute phase proteins	3
	Immunoglobulins	8
	Cytokines	9
	Phagocytic activity of lymphoid cells	1
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	2
	Phenotypic changes in lymphoid cells	4
	Weight lymphoid organs	0
	Other	9
Health status	Diarrhoea	3
	Mortality	2
	Morbility	0
	Performance	18
	Carcass traits	0
	Skeleton	0
	Other	2
	Not defined	7
Number of studies with challenge		8

3.6.1.5. Other substances

The end-points studied in porcine fed with other substances / agents are counted in Table 68.

The parameters most frequently studied with regard to local immune response were microvilli structures and intestinal microbiota (27 and 25 articles out of a total of 64, respectively), representing 42.2 and 39.0% of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was immunoglobulins (24 articles out of a total of 64), representing 37.5 % of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (50 articles out of a total of 64), representing 78.1% of the studies included in this section of the present search.

Bacteria evaluated in the different studies included pathogenic (i.e. *E. coli* and *Salmonella*) and commensal bacteria (i.e. LAB).The microvilli structure analysis includes villus height and crypt depth. The immunoglobulins evaluated include IgA, IgG, and IgM. The performance parameters evaluated included body weight, average daily gain, average daily feed intake, and feed:gain ratio.

Table 68: Number of studies for each end-point when other substances / agents are studied in porcine

PORCINE – OTHER (n = 64)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	27
	Oxidative stress	6
	Cytoquine responses	10
	Immunoglobulins	7
	Phenotypic changes in lymphoid cells	3
	Intestinal microbiota	25
	Metabolomic studies	0
	Others	22
Systemic immune response	Acute phase proteins	2
	Immunoglobulins	24
	Cytokines	15
	Phagocytic activity of lymphoid cells	2
	Cytolytic activity in lymphoid cells	1
	Lymphocyte proliferation activity	6
	Phenotypic changes in lymphoid cells	3
	Weight lymphoid organs	2
	Other	22
Health status	Diarrhoea	6
	Mortality	3
	Morbility	0
	Performance	50
	Carcass traits	2
	Skeleton	0
	Other	7
	Not defined	13
Number of studies with challenge		24

3.6.2. Poultry

As a local immune response indicator, intestinal microbiota is the parameter most frequently measured in studies that supplemented poultry diets with probiotics and plant extracts. Microvilli structure is the parameter most frequently studied in the articles in which poultry diets were supplemented with animal by-products or other substances / agents. When prebiotics are added to poultry diets, both intestinal microbiota and microvilli structure are the parameters most frequently assessed.

As a systemic immune response indicator, immunoglobulins (IgG, IgA and IgM) was the parameter most prevalent, except when plant extracts were added to poultry diets — in this case the parameter was the weight of the lymphoid organs.

The parameters used in all papers evaluating health status were related togrowth performance.

3.6.2.1. Probiotics

The end-points studied in poultry fed with probiotics are listed in Table 69.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (97 articles out of a total of 191), representing 50.8 % of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was immunoglobulins (43 articles out ofa total of 191), representing 22.5% of the total studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (128 articles out of a total of 191), representing 67.0 % of the studies included in this section of the present search. Studies of intestinal microbiota include intestinal adherence and colonisation of different bacterial strains and shedding. Bacteria evaluated in the different studies include pathogenic (i.e. *Salmonella*) and commensal bacteria (i.e. *Lactobacillus*, *Enterococcus*) or probiotics intentionally added to the diet. The immunoglobulins analysed included IgG, IgA, and IgM. The performance parameters evaluated included body weight, body weight gain, feed intake, and feed to gain ratio.

Table 69: Number of studies for each end-point when probiotics are studied in poultry

POULTRY – PROBIOTIC (n=191)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	58
	Oxidative stress	8
	Cytoquine responses	17
	Immunoglobulins	16
	Phenotypic changes in lymphoid cells	10
	Intestinal microbiota	97
	Metabolomic studies	3
	Others	42
Systemic immune response	Acute phase proteins	7
	Immunoglobulins	43
	Cytokines	7
	Phagocytic activity of lymphoid cells	7
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	5
	Phenotypic changes in lymphoid cells	2
	Weight lymphoid organs	28
	Other	39
Health status	Diarrhoea	2
	Mortality	21
	Morbility	3
	Performance	128
	Carcass traits	14
	Skeleton	0
	Other	25
	Not defined	57
Number of studies with challenge		58

3.6.2.2. Prebiotics

The end-points studied in poultry fed with prebiotics are counted in Table 70.

The parameters most frequently studied in local immune response were microvilli structures and intestinal microbiota (32 and 31 articles out of a total of 107, respectively), representing 29.9 and 29.0% of the studies included in this section of the present search. The parameter most frequently studied with regard to the systemic immune response was immunoglobulins (35 articles of a total of 107), followed by the weight of lymphoid organs (27 articles out of a total of 107), representing 32.7 and 25.2% of the studies included in this section of the present search, respectively. The parameter most frequently studied with regard to health status was performance (74 articles out of a total of 107), representing 69.2% of the studies included in this section of present search. Bacteria evaluated across the different studies include pathogenic (i.e. *Salmonella*, *E. coli*) or commensal bacteria (i.e. *Lactobacillus*, *Bifidobacteria*). A study of microvilli structures evaluates villus height and crypt depth. The

immunoglobulins analysed included IgG, IgA and IgM. The weight of lymphoid organs was measured for bursa of Fabricius, spleen, and thymus. The performance parameters evaluated included body weight, body weight gain, feed intake, and feed:gain ratio.

Table 70: Number of studies for each end-point when prebiotics are studied in poultry

POULTRY – PREBIOTIC (n=107)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	32
	Oxidative stress	5
	Cytoquine responses	13
	Immunoglobulins	5
	Phenotypic changes in lymphoid cells	4
	Intestinal microbiota	31
	Metabolomic studies	0
	Others	21
Systemic immune response	Acute phase proteins	2
	Immunoglobulins	35
	Cytokines	7
	Phagocytic activity of lymphoid cells	3
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	5
	Phenotypic changes in lymphoid cells	3
	Weight lymphoid organs	27
	Other	30
Health status	Diarrhoea	0
	Mortality	18
	Morbility	1
	Performance	74
	Carcass traits	12
	Skeleton	0
	Other	7
	Not defined	30
Number of studies with challenge		25

3.6.2.3. Plant extracts

The end-points studied in poultry fed with plant extract products are counted in Table 71.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (28 articles of a total of 74), representing 37.8% of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was the weight of lymphoid organs (28 articles out of a total of 74), representing 37.8% of the studies included in this section of the present search. The parameter most frequently studied in health status was performance (60 articles out of a total of 74),

representing 81.1% of the studies included in this section of present search. Bacteria evaluated across the different studies include pathogenic bacteria, organisms (i.e. *Salmonella*, faecal oocyst), or commensal bacteria (i.e. *Lactobacillus*). The weight of lymphoid organs was measured for bursa of Fabricius, spleen, and thymus. The performance parameters evaluated included body weight, body weight gain, feed intake and feed:gain ratio.

Table 71: Number of studies for each end-point when plant extract products are studied in poultry

POULTRY – PLANT EXTRACT (n=74)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	20
	Oxidative stress	5
	Cytoquine responses	7
	Immunoglobulins	3
	Phenotypic changes in lymphoid cells	2
	Intestinal microbiota	28
	Metabolomic studies	1
	Others	19
Systemic immune response	Acute phase proteins	2
	Immunoglobulins	23
	Cytokines	1
	Phagocytic activity of lymphoid cells	3
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	12
	Phenotypic changes in lymphoid cells	3
	Weight lymphoid organs	28
	Other	35
Health status	Diarrhoea	0
	Mortality	9
	Morbility	0
	Performance	60
	Carcass traits	13
	Skeleton	0
	Other	8
	Not defined	10
Number of studies with challenge		26

3.6.2.4. Animal by-products

The end-points studied in poultry fed with animal by-products are counted in Table 72.

The parameter most frequently studied with regard to local immune response was microvilli structure (six articles out of a total of 10), representing 60% of the studies included in this section of the present search. The parameter most frequently studied with regard to

systemic immune response was immunoglobulins (six articles out of a total of 10), representing 60% of the studies included withinthis section of present search. The parameter most frequently studied in health status was performance (six articles out ofa total of 10), representing 60% of the studies included in this section of the present search. A study of microvilli structure evaluates villus height and crypt depth. The immunoglobulins analysed included IgG, IgA and, to a minor extent, IgM. The performance parameters evaluated included body weight, body weight gain, feed intake, and feed to gain ratio.

Table 72: Number of studies for each end-point when animal by-products are studied in poultry

POULTRY – ANIMAL BY-PRODUCT (n=10)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	6
	Oxidative stress	2
	Cytoquine responses	1
	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	4
	Intestinal microbiota	4
	Metabolomic studies	0
	Others	3
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	6
	Cytokines	1
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	2
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	1
	Other	5
Health status	Diarrhoea	0
	Mortality	1
	Morbility	0
	Performance	6
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	4
Number of studies with challenge		2

3.6.2.5. Other substances

The end-points studied in poultry fed with other substances / agents are counted in Table 73.

The parameter most frequently studied with regard to local immune response was the microvilli structure (31 articles out of a total of 85), representing 36.5% of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was immunoglobulins (29 articles out of a total of 85), representing 34.1% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (49 articles out of a total of 85), representing 57.6% of the studies included in this section of the present search. A study of microvilli structure evaluates villus height and crypt depth. The immunoglobulins analysed included IgG, IgA and, to a minor extent, IgM. The performance parameters evaluated included body weight, body weight gain, feed intake, and feed to gain ratio.

Table 73: Number of studies for each end-point when other substances / agents are studied in poultry

POULTRY – OTHER (n=85)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	31
	Oxidative stress	4
	Cytoquine responses	6
	Immunoglobulins	12
	Phenotypic changes in lymphoid cells	4
	Intestinal microbiota	20
	Metabolomic studies	1
	Others	18
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	29
	Cytokines	7
	Phagocytic activity of lymphoid cells	3
	Cytolytic activity in lymphoid cells	1
	Lymphocyte proliferation activity	10
	Phenotypic changes in lymphoid cells	5
	Weight lymphoid organs	9
	Other	28
Health status	Diarrhoea	0
	Mortality	15
	Morbility	1
	Performance	49
	Carcass traits	8
	Skeleton	0
	Other	14
	Not defined	29
Number of studies with challenge		31

3.6.3. Bovine

3.6.1.1 Probiotics

The end-points studied in bovine fed with probiotics are counted in Table 74.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (10 articles out of a total of 27), representing 37.0% of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was immunoglobulins, such as IgA and IgG (seven articles out of a total of 27), representing a 25.9% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance, especially body weight (15 articles out of a total of 27), representing 55.5% of the studies included in this section of the present search.

Table 74: Number of studies for each end-point when probiotics are studied in bovine

BOVINE – PROBIOTIC (n= 27)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	2
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	2
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	10
	Metabolomic studies	0
	Others	4
Systemic immune response	Acute phase proteins	2
	Immunoglobulins	7
	Cytokines	3
	Phagocytic activity of lymphoid cells	2
	Cytolytic activity in lymphoid cells	1
	Lymphocyte proliferation activity	1
	Phenotypic changes in lymphoid cells	4
	Weight lymphoid organs	1
	Other	11
Health status	Diarrhoea	10
	Mortality	1
	Morbility	3
	Performance	15
	Carcass traits	1
	Skeleton	0
	Other	4
	Not defined	7
Number of studies with challenge		1

3.6.3.1. Prebiotics

The end-points studied in bovine fed with prebiotics are counted in Table 75.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (three articles of a total of six), representing 50 % of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was phenotypic changes in lymphoid cells (two articles out of a total of six), representing 33.3 % of the studies included within this section of the present search. The parameter most frequently studied with regard to health status was performance (three articles of a total of six), representing 50% of the studies included in this section of the present search.

Table 75: Number of studies for each end-point when prebiotics are studied in bovine

BOVINE – PREBIOTIC (n= 6)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	2
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	3
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	1
	Cytokines	1
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	2
	Weight lymphoid organs	0
	Other	2
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	3
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	3
Number of studies with challenge		1

3.6.3.2. Plant extracts

The end-points studied in bovine fed with plant extract products are counted in Table 76.

The parameters most frequently studied with regard to local immune response were microvilli structure, oxidative stress, immunoglobulins and phenotypic changes in lymphoid cells (one article each out of a total of seven) representing 14.3% of the studies included in this section of the present search. The parameter most frequently studied with regard to the systemic immune response was immunoglobulins (IgA, IgG and IgM) (three articles of a total of seven), representing 42.9% of the studies included in this section of the present search. The parameter most frequently studied in health status was performance, especially body weight and feed intake (six articles of a total of seven), representing 85.7 % of the studies included in this section of the present search.

Table 76: Number of studies for each end-point when plant extract products are studied in bovine

BOVINE – PLANT EXTRACT (n= 7)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	1
	Oxidative stress	1
	Cytoquine responses	0
	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	0
	Metabolomic studies	0
	Others	0
Systemic immune response	Acute phase proteins	2
	Immunoglobulins	3
	Cytokines	2
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	1
	Weight lymphoid organs	0
	Other	7
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	6
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	1
Number of studies with challenge		1

3.6.3.3. Animal by-products

The end-points studied in bovine fed with animal by-products are counted inTable 77.

The parameter most studied in local immune response was microvilli structure, especially villus height and crypt depth (2 articles of a total of 4), representing a 50 % of the studies included in this section of present search. The parameter most studied in systemic immune response was immunoglobulins (IgG) (3 articles of a total of 4), representing a 75 % of the studies included in this section of present search. In most articles of this section, health status was not defined (3 articles of a total of 4), representing a 75 %.

Table 77: Number of studies for each end-point when animal by-products are studied in bovine

BOVINE – ANIMAL BY-PRODUCT (n= 4)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	2
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	0
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	3
	Cytokines	1
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	0
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	3
Number of studies with challenge		0

3.6.3.4. Other substances

The end-points studied in bovine fed with other substances / agents are counted in Table 78.

The parameters most frequently studied in local immune response were intestinal microbiota and cytoquine responses (four articles each out of a total of 21), representing 19.0 % of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was immunoglobulins (eight articles out of a total

of 21), representing 38.1 % of the studies included in this section of the present search. In the majority of the studies, health status was not determined (11 articles out of a total of 21), representing 52.4 %. The parameter most frequently studied in health status was performance (seven articles out of a total of 21), representing a 33.3 % of the studies included in this section of present search.

Table 78: Number of studies for each end-point when other substances / agents are studied in bovine

BOVINE – OTHER (n= 21)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	2
	Oxidative stress	0
	Cytoquine responses	4
	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	2
	Intestinal microbiota	4
	Metabolomic studies	0
	Others	3
Systemic immune response	Acute phase proteins	1
	Immunoglobulins	8
	Cytokines	3
	Phagocytic activity of lymphoid cells	2
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	2
	Phenotypic changes in lymphoid cells	3
	Weight lymphoid organs	0
	Other	8
Health status	Diarrhoea	2
	Mortality	1
	Morbility	1
	Performance	7
	Carcass traits	0
	Skeleton	0
	Other	4
	Not defined	11
Number of studies with challenge		2

3.6.4. Ovine and caprine

3.6.4.1. Probiotics

The end-points studied in ovine and caprine populations fed with probiotics are counted in Table 79.

The parameter most frequently studied in local immune response was intestinal microbiota (five articles out of a total of 10), representing 50.0% of the studies included in this section of the present search. The parameters most frequently studied with regard to systemic

immune response were immunoglobulins and cytoquines (one articles out of a total of 10 for each), each representing 10.0% of the studies included in this section of present search. The parameter most frequently studied with regard to health status was performance (three articles out of a total of 10), representing 30.0% of the studies included in this section of the present search.

Table 79: Number of studies for each end-point when probiotics are studied in ovine and caprine

OVINE AND CAPRINE – PROBIOTIC (n= 10)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	2
	Cytoquine responses	1
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	5
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	1
	Cytokines	1
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	3
Health status	Diarrhoea	0
	Mortality	1
	Morbility	0
	Performance	3
	Carcass traits	1
	Skeleton	0
	Other	1
	Not defined	4
Number of studies with challenge		3

3.6.4.2. Prebiotics

The end-points studied in ovine and caprine fed with prebiotics are counted in Table 80.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (two articles out of a total of five), representing 40.0% of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was lymphocyte proliferation activity (one article out of a total of five), representing 20.0 % of the studies included in this section of the present search. The

parameter most frequently studied in health status was performance (three articles out of a total of five), representing 60.0% of the studies included in this section of the present search.

Table 80: Number of studies for each end-point when prebiotics are studied in ovine and caprine

OVINE AND CAPRINE – PREBIOTIC (n =5)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	2
	Metabolomic studies	0
	Others	0
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	1
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	4
Health status	Diarrhoea	1
	Mortality	1
	Morbility	0
	Performance	3
	Carcass traits	1
	Skeleton	0
	Other	1
	Not defined	2
Number of studies with challenge		0

3.6.4.3. Plant extracts

The end-points studied in ovine and caprine fed with plant extract products are counted in Table 81.

Only one study was found in this section. The parameter studied with regard to local immune response was oxidative stress; the parameters studied in systemic immune response were acute phase proteins and immunoglobulins, and the parameter studied in health status was "other substances" (blood plasma metabolites).

Table 81: Number of studies for each end-point when plant extract products are studied in ovine and caprine

OVINE AND CAPRINE – PLANT EXTRACT (n = 1)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	1
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	0
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	1
	Immunoglobulins	1
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	1
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	0
Number of studies with challenge		0

3.6.4.4. Animal by-products

The end-points studied in ovine and caprine fed with animal by-products are counted in Table 82.

Only one study was included in this section. The parameter studied in local immune response was intestinal microbiota. Parameters related to systemic immune response and health status were not measured.

Table 82: Number of studies for each end-point when animal by-products are studied in ovine and caprine

OVINE AND CAPRINE– ANIMAL BY-PRODUCT (n =1)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0

	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	1
	Metabolomic studies	0
	Others	0
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	0
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	1
Number of studies with challenge		1

3.6.4.5. Other substances

The end-points studied in ovine and caprine populations fed with other substances / agents are counted in Table 83.

The parameters most frequently studied with regard tolocal immune response were intestinal microbiota (two articles each of a total of 4), representing 50.0% of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was cytokines (two articles out of a total of four), representing 50.0% of the studies included in this section of the present search. The parameter most frequently studied in health status was performance (three articles out of a total of four), representing 75.0% of the studies included in this section of the present search.

Table 83: Number of studies for each end-point when other substances / agents are studied in ovine and caprine

OVINE AND CAPRINE – OTHER (n = 4)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	1
	Cytoquine responses	1

	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	2
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	2
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	1
	Weight lymphoid organs	0
	Other	2
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	3
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	1
Number of studies with challenge		0

3.6.5. Fish

3.6.5.1. Fish (Salmonids)

3.6.5.1.1. Probiotics

The end-points studied in fish (group 7.1. Salmonids) fed with probiotics are counted in Table 84.

The immunological parameter most frequently studied was lysozyme activity (19 articles out of a total of 39), representing 48.7% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was mortality (14 articles out of a total of 39), representing 35.9 % of the studies included in this section of the present search.

Table 84: Number of studies for each end-point when probiotics are studied in fish (Salmonids)

FISH (Salmonids) - PROBIOTIC (n = 39)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	10
	Complement activity	12
	Leucocyte count	11

	Immunoglobulin quantification	8
	Haematocrit	6
	Respiratory (oxidative) burst	13
	Phosphatase activity	1
	Protease activity	6
	Phenoloxidase activity	0
	Lysozyme activity	19
	Peroxidase activity	4
	Bacterial activity	9
	Intestine morphology	6
	Other	30
Health status	Diarrhoea	1
	Mortality	14
	Morbility	0
	Performance	12
	Carcass traits	3
	Skeleton	0
	Other	0
	Not defined	21
Number of studies with challenge		17

3.6.5.1.2. Prebiotics

The end-points studied in fish (group 7.1. Salmonids) fed with prebiotics are counted in Table 85.

The immunological parameter most frequently studied was intestine morphology (five articles out of a total of 14), representing 35.7% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (five articles out of a total of 14), representing 35.7% of the studies included in this section of the present search.

Table 85: Number of studies for each end-point when prebiotics are studied in fish (Salmonids)

FISH – PREBIOTIC (Salmonids) (n=14)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	4
	Complement activity	3
	Leucocyte count	1
	Immunoglobulin quantification	4
	Haematocrit	1
	Respiratory (oxidative) burst	2
	Phosphatase activity	0
	Protease activity	0
	Phenoloxidase activity	0
	Lysozyme activity	3
	Peroxidase activity	0
	Bacterial activity	1

	Intestine morphology	5
	Other	11
Health status	Diarrhoea	1
	Mortality	3
	Morbility	0
	Performance	5
	Carcass traits	1
	Skeleton	0
	Other	0
	Not defined	7
Number of studies with challenge		4

3.6.5.1.3. Plant extracts

The end-points studied in fish (group 7.1. Salmonids) fed with plant extract products are counted in Table 86.

The immunological parameter most frequently studied was respiratory burst activity (five articles of a total of five), representing 100% of the studies included in this section of the present search. The parameter most frequently studied in health status was performance such as body weight and growth rate (three articles out of a total of five), representing 60% of the studies included in this section of the present search.

Table 86: Number of studies for each end-point when plant extract products are studied in fish (Salmonids)

FISH – PLANT EXTRACT (Salmonids) (n=5)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	3
	Complement activity	2
	Leucocyte count	2
	Immunoglobulin quantification	1
	Haematocrit	2
	Respiratory (oxidative) burst	5
	Phosphatase activity	0
	Protease activity	1
	Phenoloxidase activity	0
	Lysozyme activity	3
	Peroxidase activity	3
	Bacterial activity	1
	Intestine morphology	1
	Other	3
Health status	Diarrhoea	0
	Mortality	1
	Morbility	0
	Performance	3

	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	1
Number of studies with challenge		2

3.6.5.1.4. Animal by-products

The end-points studied in fish (group 7.1. Salmonids) fed with animal by-products are counted in Table 87.

In this section, only one study for group 7.1. Salmonids was identified. The immunological parameters evaluated in this study were complement activity, lysozyme activity, and peroxidise activity. Diarrhoea and morbidity were the parameters studied with regard to health status.

Table 87: Number of studies for each end-point when animal by-productsare studied in fish (Salmonids)

FISH – ANIMAL BY-PRODUCT (Salmonids) (n = 1)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	0
	Complement activity	1
	Leucocyte count	0
	Immunoglobulin quantification	0
	Haematocrit	0
	Respiratory (oxidative) burst	0
	Phosphatase activity	0
	Protease activity	0
	Phenoloxidase activity	0
	Lysozyme activity	1
	Peroxidase activity	1
	Bacterial activity	0
	Intestine morphology	0
	Other	0
Health status	Diarrhoea	1
	Mortality	0
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	0
Number of studies with challenge		1

3.6.5.1.5. Other substances

The end-points studied in fish (group 7.1. Salmonids) fed with other substances / agents are counted in Table 88.

The immunological parameter most frequently studied was respiratory (oxidative) burst (15 articles out of a total of 26), representing 57.7% of the studies included in this section of the present search. The parameter most frequently studied in health status was performance such as body weight and growth rate (14 articles out of a total of 26), representing 53.8% of the studies included in this section of the present search.

Table 88: Number of studies for each end-point when other substances / agents are studied in fish (Salmonids)

FISH – OTHER (Salmonids) (n = 26)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	11
	Complement activity	6
	Leucocyte count	0
	Immunoglobulin quantification	6
	Haematocrit	1
	Respiratory (oxidative) burst	15
	Phosphatase activity	2
	Protease activity	2
	Phenoloxidase activity	3
	Lysozyme activity	11
	Peroxidase activity	4
	Bacterial activity	3
	Intestine morphology	7
	Other	19
Health status	Diarrhoea	0
	Mortality	10
	Morbility	0
	Performance	14
	Carcass traits	1
	Skeleton	0
	Other	1
	Not defined	5
Number of studies with challenge		7

3.6.5.2. Fish (Freshwater fish)

3.6.5.2.1. Probiotics

The end-points studied in fish (group 7.2. Freshwater fish) fed with probiotics are counted in Table 89.

The immunological parameter most frequently studied was lysozyme activity (23 articles out of a total of 42), representing 54.7% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was mortality (19 articles of a total of 42), representing 45.2 % of the studies included in this section of the present search.

Table 89: Number of studies for each end-point when probiotics are studied in fish (Freshwater fish)

FISH (freshwater) - PROBIOTIC (n =42)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	13
	Complement activity	15
	Leucocyte count	13
	Immunoglobulin quantification	9
	Haematocrit	11
	Respiratory (oxidative) burst	20
	Phosphatase activity	1
	Protease activity	0
	Phenoloxidase activity	1
	Lysozyme activity	23
	Peroxidase activity	5
	Bacterial activity	3
	Intestine morphology	4
	Other	32
Health status	Diarrhoea	0
	Mortality	19
	Morbility	0
	Performance	17
	Carcass traits	0
	Skeleton	0
	Other	4
	Not defined	13
Number of studies with challenge		24

3.6.5.2.2. Prebiotics

The end-points studied in fish (group 7.2. freshwater fish) fed with prebiotics are counted in Table 90.

The immunological parameter most frequently studied was lysozyme activity (12 articles out of a total of 34), representing 35.2% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (18 articles out of a total of 34), representing 52.9% of the studies included in this section of the present search.

Table 90: Number of studies for each end-point when prebiotics are studied in fish (Freshwater fish)

FISH – PREBIOTIC (freshwater) (n=34)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	8
	Complement activity	6
	Leucocyte count	10
	Immunoglobulin quantification	8
	Haematocrit	8
	Respiratory (oxidative) burst	11
	Phosphatase activity	1
	Protease activity	0
	Phenoloxidase activity	1
	Lysozyme activity	12
	Peroxidase activity	2
	Bacterial activity	2
	Intestine morphology	6
	Other	31
Health status	Diarrhoea	0
	Mortality	13
	Morbility	0
	Performance	18
	Carcass traits	4
	Skeleton	0
	Other	1
	Not defined	12
Number of studies with challenge		14

3.6.5.2.3. Plant extracts

The end-points studied in fish (group 7.2. Freshwater fish) fed with plant extract products are counted in Table 91.

The immunological parameter most frequently studied was lysozyme activity (seven articles out of a total of 11), representing 63.6% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance, such as body weight and growth rate (five articles out of a total of 11), representing 45.4% of the studies included in this section of the present search.

Table 91: Number of studies for each end-point when plant extract products are studied in fish (Freshwater fish)

FISH – PLANT EXTRACT (freshwater) (n=11)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	3
	Complement activity	1
	Leucocyte count	3
	Immunoglobulin quantification	1
	Haematocrit	4
	Respiratory (oxidative) burst	5
	Phosphatase activity	0
	Protease activity	0
	Phenoloxidase activity	0
	Lysozyme activity	7
	Peroxidase activity	0
	Bacterial activity	2
	Intestine morphology	1
	Other	6
Health status	Diarrhoea	0
	Mortality	2
	Morbility	0
	Performance	5
	Carcass traits	1
	Skeleton	0
	Other	1
	Not defined	4
Number of studies with challenge		5

3.6.5.2.4. Animal by-products

The end-points studied in fish (group 7.2. freshwater fish) fed with animal by-products are counted in Table 92.

In this section, only one study for the group 7.2. freshwater fish was identified. The immunological parameters evaluated in this study were complement activity, lysozyme activity, and peroxidise activity. Mortality and performance were the parameters studied with regard to health status.

Table 92: Number of studies for each end-point when animal by-products are studied in fish (Freshwater fish)

FISH – ANIMAL BY-PRODUCT (freshwater) (n =2)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	0
	Complement activity	0
	Leucocyte count	0
	Immunoglobulin quantification	1
	Haematocrit	0
	Respiratory (oxidative) burst	1

	Phosphatase activity	0
	Protease activity	0
	Phenoloxidase activity	0
	Lysozyme activity	1
	Peroxidase activity	0
	Bacterial activity	0
	Intestine morphology	0
	Other	1
Health status	Diarrhoea	0
	Mortality	2
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	0
Number of studies with challenge		2

3.6.5.2.5. Other substances

The end-points studied in fish (group 7.2. freshwater fish) fed with other substances / agents are counted in Table 93.

The immunological parameter most frequently studied was lysozyme activity (20 articles out of a total of 96), representing 20.8% of the studies included in this section of present search. The parameter most frequently studied with regard to health status was performance, such as body weight and growth rate (19 articles out of a total of 96), representing 19.7 % of the studies included in this section of the present search.

Table 93: Number of studies for each end-point when other substances / agents are studied in fish (Freshwater fish)

FISH – OTHER (freshwater) (n =96)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	9
	Complement activity	10
	Leucocyte count	6
	Immunoglobulin quantification	14
	Haematocrit	7
	Respiratory (oxidative) burst	14
	Phosphatase activity	4
	Protease activity	1
	Phenoloxidase activity	0
	Lysozyme activity	20
	Peroxidase activity	4
	Bacterial activity	3
	Intestine morphology	4
	Other	24

Health status	Diarrhoea	0
	Mortality	16
	Morbility	0
	Performance	19
	Carcass traits	1
	Skeleton	0
	Other	1
	Not defined	6
Number of studies with challenge		16

3.6.5.3. Fish (Marine fish and Shellfish)

3.6.5.3.1. Probiotics

The end-points studied in fish (group 7.3. Marine Fish and Shellfish) fed with probiotics are counted in Table 94.

The immunological parameter most frequently studied was respiratory (oxidative) burst (49 articles out of a total of 94), representing 52.1 % of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was mortality (56 articles out of a total of 94), representing 59.5 % of the studies included in this section of the present search.

Table 94: Number of studies for each end-point when probiotics are studied in fish (Marine fish and Shellfish)

FISH (Marine Fish and Shellfish) - PROBIOTIC (n = 94)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	42
	Complement activity	18
	Leucocyte count	11
	Immunoglobulin quantification	14
	Haematocrit	4
	Respiratory (oxidative) burst	49
	Phosphatase activity	10
	Protease activity	8
	Phenoloxidase activity	19
	Lysozyme activity	29
	Peroxidase activity	17
	Bacterial activity	8
	Intestine morphology	12
	Other	71
Health status	Diarrhoea	0
	Mortality	56
	Morbility	0
	Performance	46
	Carcass traits	1

	Skeleton	1
	Other	3
	Not defined	23
Number of studies with challenge		49

3.6.5.3.2. Prebiotics

The end-points studied in fish (group 7.3. marine fish and shellfish) fed with prebiotics are counted in Table 95.

The immunological parameters most frequently studied were phagocityc activity and respiratory (oxidative) burst for both endpoints (13 articles out of a total of 40), representing 28.8% for each indicator of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (26 articles out of a total of 40), representing 65.0% of the studies included in this section of the present search.

Table 95: Number of studies for each end-point when prebiotics are studied in fish (Marine fish and Shellfish)

FISH – PREBIOTIC (Marine Fish and Shellfish) (n=40)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	13
	Complement activity	9
	Leucocyte count	2
	Immunoglobulin quantification	2
	Haematocrit	2
	Respiratory (oxidative) burst	13
	Phosphatase activity	3
	Protease activity	2
	Phenoloxidase activity	7
	Lysozyme activity	11
	Peroxidase activity	3
	Bacterial activity	0
	Intestine morphology	11
	Other	25
Health status	Diarrhoea	0
	Mortality	20
	Morbility	0
	Performance	26
	Carcass traits	0
	Skeleton	0
	Other	4
	Not defined	10
Number of studies with challenge		14

3.6.5.3.3. Plant extracts

The end-points studied in fish (group 7.3. marine fish and shellfish) fed with plant extract products are counted in Table 96.

The immunological parameter most frequently studied was lysozyme activity (16 articles out of a total of 27), representing 59.2% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was mortality (16 articles out of a total of 27), representing 59.2% of the studies included in this section of the present search.

Table 96: Number of studies for each end-point when plant extract products are studied in fish (Marine fish and Shellfish)

FISH – PLANT EXTRACT (Marine Fish and Shellfish) (n=27)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	9
	Complement activity	7
	Leucocyte count	6
	Immunoglobulin quantification	2
	Haematocrit	6
	Respiratory (oxidative) burst	14
	Phosphatase activity	2
	Protease activity	3
	Phenoloxidase activity	3
	Lysozyme activity	16
	Peroxidase activity	7
	Bacterial activity	7
	Intestine morphology	0
	Other	20
Health status	Diarrhoea	0
	Mortality	16
	Morbility	0
	Performance	15
	Carcass traits	2
	Skeleton	0
	Other	0
	Not defined	5
Number of studies with challenge		18

3.6.5.3.4. Animal by-products

The end-points studied in fish (group 7.3. Marine Fish and Shellfish) fed with animal by-products are counted in Table 97.

In this section, only 11 studies for group 7.3. Marine Fish and Shellfish were identified. The immunological parameters evaluated in this group were not clear defined. Mortality and performance were the parameters studied with regard tohealth status.

Table 97: Number of studies for each end-point when animal by-products are studied in fish (Marine fish and Shellfish)

FISH – ANIMAL BY-PRODUCT (Marine Fish and Shellfish) (n =11)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	1
	Complement activity	1
	Leucocyte count	0
	Immunoglobulin quantification	1
	Haematocrit	0
	Respiratory (oxidative) burst	2
	Phosphatase activity	1
	Protease activity	0
	Phenoloxidase activity	0
	Lysozyme activity	1
	Peroxidase activity	1
	Bacterial activity	0
	Intestine morphology	0
	Other	3
Health status	Diarrhoea	0
	Mortality	3
	Morbility	0
	Performance	2
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	0
Number of studies with challenge		2

3.6.5.3.5. Other substances

The end-points studied in fish (group 7.3. Marine Fish and Shellfish) fed with other substances / agents are counted in Table 98.

The immunological parameter most frequently studied was lysozyme activity (15 articles out of a total of 26), representing 68.7% of the studies included within this section of the present search. The parameter most frequently studied with regard to health status was performance, such as body weight and growth rate (14 articles out of a total of 26), representing 53.8% of the studies included within this section of the present search.

Table 98: Number of studies for each end-point when other substances / agents are studied in fish (Marine fish and Shellfish)

FISH – OTHER (Marine Fish and Shellfish) (n =26)		Number of articles
Immunological parameters studied (fish)	Phagocytic activity	11
	Complement activity	6
	Leucocyte count	0
	Immunoglobulin quantification	6
	Haematocrit	1
	Respiratory (oxidative) burst	15
	Phosphatase activity	2
	Protease activity	2
	Phenoloxidase activity	3
	Lysozyme activity	11
	Peroxidase activity	4
	Bacterial activity	3
	Intestine morphology	7
	Other	19
Health status	Diarrhoea	0
	Mortality	10
	Morbility	0
	Performance	14
	Carcass traits	1
	Skeleton	0
	Other	1
	Not defined	5
Number of studies with challenge		7

3.6.6. Rabbits

With regard to the parameters used for the evaluation of the effects of feed additives on rabbits, the most frequently used are productive parameters: body weight gain (BWG), feed intake (FI), and the weight gain/feed intake ratio (FCR) or derived parameters (ADG, ADFI). In addition, mortality rate is the most frequently repeated indicator of the evaluation of the health status of animals and the reduction of the mortality is the principal marker for health improvement induced by the examined feed additives.

Another indicator, in this case related to gut health, is the study of the intestinal mucosa morphology, specially the microvilli high (VH), crypt depth (CD), and ratio VH/CD. The analysis of the intestinal mucosa structure was used in five papers.

As direct measurement of the immune system status, intra-epithelial lymphocytes (IEL) were measured in only one study; where lymphocyte subpopulations ($CD4^+$, $CD5^+$, $CD8^+$, $CD4^+CD8^+$, $CD25^+$, $CD5^+CD25^+$) were determined as well. In one study, the quantification of

the phagocytic activity of leucocytes was used as an evaluator of natural immunity. The appendix weight and the number and size of the Peyer's patches and follicles presented in both structures are additional parameters used for the evaluation of the intestinal immune system.

The anti-inflammatory effect of feed additives was evaluated by IL-2, myeloperoxidase (MPO) and peroxisome proliferator-activated receptor (PPAR) quantification in two different studies.

The effects of feed additives on intestinal bacteria translocation to MLNs, liver, spleen, and lung were examined in two articles. The bacterial translocation can be considered the combined effect of the integrity degree of intestinal mucosa and the status of the natural / acquired immunity.

Finally, the last effect of feed additives examined in some of the reviewed papers is the change in intestinal microbiota. Seven articles examined these changes, two using general microbiota profiles by PCR-restriction fragment length polymorphism (RFLP) techniques; another five counted specific microorganisms such as *Bacteroides spp.*, *Escherichia coli*, *Fibrobacter succinogenes*, *Pseudomonas spp., Salmonella enterica, Clostridium perfringens*, *Clostridium spiroforme*, *Enterococcus faecium*, lactic acid bacteria or *Staphylococcus aureus*.

3.6.6.1. Probiotics

The end-points studied in rabbits fed with probiotics are counted in Table 99.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (seven articles of a total of nine), representing 77.8% of the studies included in this section of the present search. The parameter most studied in systemic immune response was phagocytic activity of lymphoid cells (one article out of a total of nine), each representing 11.1% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (five articles of a total of nine), representing 55.5% of the studies included in this section of the present search.

Table 99: Number of studies for each end-point when probiotics are studied in rabbits

RABBITS – PROBIOTIC (n = 9)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	3
	Oxidative stress	1
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	7
	Metabolomic studies	0
	Others	2
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	1
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	2
Health status	Diarrhoea	0
	Mortality	2
	Morbility	0
	Performance	5
	Carcass traits	0
	Skeleton	0
	Other	2
	Not defined	3
Number of studies with challenge		2

3.6.6.2. Prebiotics

The end-points studied in rabbits fed with prebiotics are counted in Table 100.

There is only one study in this section. The parameters studied with regard to local immune response were microvilli structure and intestinal microbiota. Parameters in systemic immune response were not studied. The parameter studied with regard to health status was performance.

Table 100: Number of studies for each end-point when prebiotics are studied in rabbits

RABBITS – PREBIOTIC (n = 1)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	1
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	1
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0

	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	0
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	0
Number of studies with challenge		0

3.6.6.3. Plant extracts

The end-points studied in rabbits fed with plant extracts are counted in Table 101.

The parameters most frequently studied with regard to local immune response were intestinal microbiota and phenotypic changes in lymphoid cells (two articles out of a total of three each), representing 66.7% of the studies included in this section of the present search. The parameter studied in systemic immune response was phagocytic activity of lymphoid cells (one article of a total of three), each representing 33.3% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was mortality (two articles of a total of three), representing 66.7% of the studies included in this section of present search.

Table 101: Number of studies for each end-point when plant extracts are studied in rabbits

RABBITS – PLANT EXTRACT (n = 3)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	1
	Oxidative stress	1
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	2
	Intestinal microbiota	2
	Metabolomic studies	0
	Others	2
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	1

	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	0
Health status	Diarrhoea	0
	Mortality	2
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	1
Number of studies with challenge		0

3.6.6.4. Animal by-products

Studies were not found in this section.

3.6.6.5. Other substances

The end-points studied in rabbits fed with other substances / agents are counted in Table 102.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (three articles of a total of three each), representing 100% of the studies included in this section of the present search. The parameter studied with regard to systemic immune response was immunoglobulins (one articles of a total of three), representing 33.3% of the studies included in this section of the present search. The parameter most frequently studied with regard to health status was performance (three articles of a total of three) representing 100% of the studies included in this section of the present search.

Table 102: Number of studies for each end-point when other substances / agents are studied in rabbits

RABBITS – OTHER (n = 3)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	3
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0

	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	1
	Metabolomic studies	0
	Others	2
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	1
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	1
Health status	Diarrhoea	0
	Mortality	2
	Morbility	0
	Performance	3
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	0
Number of studies with challenge		1

3.6.7. Equine

3.6.7.1. Probiotics

The end-points studied in equine fed with probiotics are counted in Table 103.

The parameter most frequently studied with regard tolocal immune response was intestinal microbiota (twoarticles out ofa total of four), representing 50.0 % of the studies included in this section of the present search. The parameter most frequently studied in systemic immune response was other (leucopaenia) (onearticle of a total of four), each representing 25.0 % of the studies included in this section of the present search. The parameter most frequently studied with regard tohealth status was performance (onearticles of a total of four), representing 25.0 % of the studies included in this section of the present search.

Table 103: Number of studies for each end-point when probiotics are studied in equine populations

EQUINE – PROBIOTIC (n = 4)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	2

	Metabolomic studies	0
	Others	2
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	1
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	2
Number of studies with challenge		0

3.6.7.2. Prebiotics

The end-points studied in equine fed with prebiotics are counted in Table 104.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (one article of a total of two), representing 50.0% of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was cytokines (one article out of a total of two), each representing 50.0% of the studies included in this section of the present search. Health status was not defined in any of the studies (two articles out of a total of two) representing 100% of the studies included in this section of the present search.

Table 104: Number of studies for each end-point when prebiotics are studied in equine populations

EQUINE – PREBIOTIC (n = 2)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	1
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	1

	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	1
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	2
Number of studies with challenge		1

3.6.7.3. Plant extracts

The end-points studied in equine fed with plant extracts are counted in Table 105.

This section only includes one study. The parameter studied in local immune response was other (intestine inflammation). Parameters with regard to systemic immune response were not studied. The parameter studied with regard to health status was other (intestinal motility).

Table 105: Number of studies for each end-point when plant extracts are studied in equine populations

EQUINE – PLANT EXTRACT (n = 1)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	0
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	0
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0

	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	0
Number of studies with challenge		1

3.6.7.4. Animal by-products

No studies were found in this section.

3.6.7.5. Other substances

The end-points studied in equine fed with other substances / agents are counted in Table 106. Local immune response was not studied. The parameters most frequently studied in systemic immune response were immunoglobulins, cytokines, and lymphocyte proliferation activity (one article for each parameter, with a total of two articles), representing 50.0% of the studies included in this section of the present search. Health status was not defined here.

Table 106: Number of studies for each end-point when other substances / agents are studied in equine

EQUINE – OTHER (n = 2)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	0
	Metabolomic studies	0
	Others	0
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	1
	Cytokines	1
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	1
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	1
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0

	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	2
Number of studies with challenge		0

3.6.8. Pets

3.6.8.1. Probiotics

The end-points studied in pets fed with probiotics are counted in Table 107.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (14 articles of a total of 21), representing 66.7% of the studies included in this section of the present search. The parameter most frequently studied was immunoglobulins (three articles of a total of 21), each representing 14.3% of the studies included in this section of the present search. In most of studies, health status was not defined (16 articles out of a total of 21), representing 76.2% of the studies included in this section of the present search.

Table 107: Number of studies for each end-point when probiotics are studied in pets

PETS – PROBIOTIC (n = 21)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	1
	Cytoquine responses	3
	Immunoglobulins	3
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	14
	Metabolomic studies	0
	Others	3
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	3
	Cytokines	1
	Phagocytic activity of lymphoid cells	1
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	1
	Phenotypic changes in lymphoid cells	1
	Weight lymphoid organs	0
	Other	2
Health status	Diarrhoea	3
	Mortality	0
	Morbility	1
	Performance	3
	Carcass traits	0
	Skeleton	0

	Other	0
	Not defined	16
Number of studies with challenge		1

3.6.8.2. Prebiotics

The end-points studied in pets fed with prebiotics are counted in Table 108.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (13 articles out of a total of 17), representing 94.1% of the studies included in this section of the present search. The parameter most frequently studied with regard to systemic immune response was immunoglobulins (six articles of a total of 17), each representing 35.3% of the studies included in this section of the present search. In most of these studies, health status was not defined (nine articles out of a total of 17), representing 52.9 % of the studies included in this section of the present search.

Table 108: Number of studies for each end-point when prebiotics are studied in pets

PETS – PREBIOTIC (n = 17)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	1
	Oxidative stress	1
	Cytoquine responses	0
	Immunoglobulins	5
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	13
	Metabolomic studies	1
	Others	3
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	6
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	6
Health status	Diarrhoea	4
	Mortality	1
	Morbility	1
	Performance	4
	Carcass traits	0
	Skeleton	0
	Other	3
	Not defined	9
Number of studies with challenge		1

3.6.8.3. Plant extracts

The end-points studied in pets fed with plant extracts are counted in Table 109.

The parameter most frequently studied with regard to local immune response was intestinal microbiota (three articles out of a total of three each), representing 100% of the studies included in this section of the present search. The parameter studied in systemic immune response was acute phase proteins (two articles of a total of three), each representing 66.7 % of the studies included in this section of the present search. In most studies, health status was not defined (two articles of a total of three), representing 66.7% of the studies included in this section of the present search.

Table 109: Number of studies for each end-point when plant extracts are studied in pets

PETS – PREBIOTIC (n = 3)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	0
	Intestinal microbiota	3
	Metabolomic studies	0
	Others	1
Systemic immune response	Acute phase proteins	2
	Immunoglobulins	0
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	1
	Weight lymphoid organs	0
	Other	2
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	1
	Not defined	2
Number of studies with challenge		0

3.6.8.4. Animal by-products

The end-points studied in pets fed with animal by-products are counted in Table 110.

Only one study was included in this section. The parameters studied in local immune response were immunoglobulins and phenotypic changes in lymphoid cells. The parameter studied in systemic immune response was immunoglobulins. Parameters related with health status were not measured.

Table 110: Number of studies for each end-point when animal by-products are studied in pets

PETS – ANIMAL BY-PRODUCT (n =1)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	0
	Immunoglobulins	1
	Phenotypic changes in lymphoid cells	1
	Intestinal microbiota	0
	Metabolomic studies	0
	Others	0
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	1
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	0
	Lymphocyte proliferation activity	0
	Phenotypic changes in lymphoid cells	0
	Weight lymphoid organs	0
	Other	0
Health status	Diarrhoea	0
	Mortality	0
	Morbility	0
	Performance	0
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	1
Number of studies with challenge		0

3.6.8.5. Other substances

The end-points studied in pets fed with other substances / agents are counted in Table 111.

The parameters most frequently studied with regard to local immune response were cytoquine responses and phenotypic changes in lymphoid cells (two articles for each parameter, out of a total of four articles), representing 50.0% of the studies included in this section of the present search. The parameters most frequently studied with regard to systemic immune response were immunoglobulins, cytolytic activity in lymphoid cells, lymphocyte proliferation activity, and phenotypic changes in lymphoid cells (one article for

each parameter out of a total of four), representing 25.0% of the studies included in this section of the present search. In half of the articles, health status was not defined (two out of a total of 4 articles), representing 50% of the studies included in this section of the present search.

Table 111: Number of studies for each end-point when other substances / agents are studied in pets

PETS – OTHER (n = 4)		Number of articles
Local immune response	Microvilli structure: length of microvilli and crypts	0
	Oxidative stress	0
	Cytoquine responses	2
	Immunoglobulins	0
	Phenotypic changes in lymphoid cells	2
	Intestinal microbiota	1
	Metabolomic studies	0
	Others	0
Systemic immune response	Acute phase proteins	0
	Immunoglobulins	1
	Cytokines	0
	Phagocytic activity of lymphoid cells	0
	Cytolytic activity in lymphoid cells	1
	Lymphocyte proliferation activity	1
	Phenotypic changes in lymphoid cells	1
	Weight lymphoid organs	0
	Other	1
Health status	Diarrhoea	1
	Mortality	0
	Morbility	0
	Performance	1
	Carcass traits	0
	Skeleton	0
	Other	0
	Not defined	2
Number of studies with challenge		0

3.6.9. Methods for the objective measurement of end-points: potential use and limitations

The following tables illustrate the relationship between end-points and methodology in order to analyse each immunological parameter. This information provides an understanding of the limitations and the potential uses of the most important end-points. These parameters are usually assessed in order to characterise the immune response induced by the immunomodulatory substances / agents identified in terrestrial animals (Table 112) and in fish and shellfish (Table 113).

Table 112: Summary of the principal end-points used for the efficacy evaluation of immunomodulatory substances / agents in terrestrial animals

	PARAMETERS	AIM/SIGNIFICANCE	METHODOLOGY	UNITS	COMMENTS
Performance					
	Body Weight Increase (BWI)	Basic performance data. In general, an increase of BWI is associated with positive effects of tested products.	Calibrated balance	Difference of individual body weight at times, in kg	Easy collection.
	Feed intake (FI)	Basic performance data. A reduction of FI associated with the maintenance of BW; this is associated with positive effects of tested products.	Calibrated balance	Weight of the feed consumed at different times, in kg	The individual measurement of feed intake is not possible under normal experimental conditions. Frequently the measure is collective for the animals into each experimental group.
	Feed Conversion Ratio (FCR)	Parameter that integrates BW and FI parameters. It is the principal indicator for animal performance.	Mathematical calculation	FI/BWI	The accuracy of this data depends on the accuracy of the feed intake measurement.
	Daily Weigh Gain (DWG)	An increase of DWG is associated with positive effects of tested products.	Mathematical calculation	BWI/days	Easy calculation.
	Daily Feed Intake (DFI)	A reduction of DFI is associated with the maintenance of DBW as well as with positive effects of tested products.	Mathematical calculation	FI/days	Easy calculation.
Pathology					
	Clinical signs	Under field or experimental conditions, the evaluation of clinical signs is used for the evaluation of the health status of animals.	Direct observation	% of animals	Type and scale must be clearly defined in the experimental protocol.
	Mortality	Primary indicator for the evaluation of the health status of animals.	Direct observation	% of animals	Easy collection. It is necessary to determine the causes.
Histopathology					
	Microvilli high	Gut health and digestive improvement is associated to an increase in microvilli high.	Staining and microscopy	µm (average of ten measurements per sample)	Easy collection but time consuming.
	Crypt depth	An increase is associated with intestinal mucosa regeneration following epithelial damage.	Staining and microscopy	µm (average of ten measurements per sample)	Easy collection but time consuming.
	Intraepithelial lymphocytes	The persistence of inflammatory conditions at gut level is associated with the increase	Staining and microscopy	Number of cells (average of ten	Easy collection but time consuming.

		of intraepithelial lymphocytes.		measurements per sample)	
	Goblet cell	The persistence of inflammatory conditions at gut level is associated with the increase of goblet cells.	Staining and microscopy	Number and type (average of ten measurements per sample)	Easy collection but time consuming.
Microbiology					
	Presence/absence	The specific detection of some bacterial species (e.g. *Escherichia coli* K88, *Salmonella enterica*, *Clostridium perfringens*, *Brachyspira hyodysenteriae*, etc.) is used for the evaluation of enteric pathogen control induced for tested products and the improvement of gut health.	Bacterial culture on selective media	% of positive samples	It is necessary the use of selective media or specific primers.
	Bacterial quantification	The quantification of some bacterial species (e.g. *Escherichia coli* K88, *Salmonella enterica*, *Clostridium perfringens*, *Brachyspira hyodysenteriae,* etc.) is used for the evaluation of enteric pathogen control induced for tested products and the improvement of gut health.	Plate count	CFU/weight or volume of sample	Easy but time consuming.
			qRT-PCR	CFU/weight or volume of sample	Special equipment is necessary. Standard and controls are necessary. The non-viable bacteria are also determined.
	Study of bacterial population	Gut health is associated with specific microbiota profiles. Dysbiosis is related to negative performance and, in some cases,to digestive disorders.	DGGE or PCR-RFLP	Electrophoretic profiles	Easy but reduced specificity.
			Deep sequencing	OTUs quantification	The most accurate method. Special equipment and software are necessary. Expensive.
	Bacterial metabolism	In general, the increase of beneficial microbiota components is associated with an increase of volatile fatty acids (VFA), such asacetic acid (reducing faecal excretion of Ca and magnesium (Mg)), propionic acid (increase the intestinal absorption of water and nutrients), lactic acid (reduction of pH and antibacterial effect), and butyric acid (increase the integrity of the intestinal mucosa)).	Normalized chemical methods	mg or moles/ weight or volume of sample	The collection and conservation of samples are the most critical point. Easy determination.

	Bacterial translocation	Indirect measurement for the integrity of intestinal mucosa. Liver, spleen or MLNs translocation are frequently examined.	Microbiological methods	CFU/weight of sample	Easy but time consuming.
Immunology					
	Weight/size of lymphoid organs	The measurement of appendix and Peyer's patches are indicators of the general stimulation of GALT (gut associated lymphoid tissue).	Calibrated balance and caliper.	g/mm	Easy but time consuming.
	Immunoglobulins	The quantification of general or specific immunoglobulins is used for the evaluation of the improvement of humoral immune response by tested products.	ELISA	mg/mL	Easy, low-cost.
	Specific surface antigens	The study of surface antigens is necessary to analyse the immune-system components improved by tested products. Some of the examined markers are: MHC-II (associated to antigen presenting cells, like macrophages or dendritic cells), TLR-2 (associated with Gram-positive bacteria recognition and activation of innate immunity), TLR-4 (associated with the recognition of Gram-negative lipopolysaccharide and activation of innate immunity), CD2 (associated to T-cells and NK cells), CD4 (associated to T-helper cells), CD8 (associated to cytotoxic T-cells), CD5 (associated to IgM-secreting B-cells and T-cells), CD25 (associated to activated T-cells, activated B-cells, some thymocytes, myeloid precursors).	Immunohistochemistry	Number of labelled cells/total cells	The use of monoclonal antibodies is necessary. Commercial reagents are easily obtained. Time consuming and expensive.
			Confocal microscopy	Number of labelled cells/total cells	Special equipment and software are necessary. Very accurate analysis. Time consuming and expensive.
			Flow cytometry	Number of labelled cells/total cells	Special equipment and software are necessary. Very accurate analysis. Time consuming and expensive.
	Cytokine production	Small proteins are important to cell signalling during immune response. Some of these are related to cellular immune response type 1 (IFN-γ, TNF-α), and some others with cellular immune response type 2 (TGF-β, IL-4, IL-10, IL-13).	ELISA	µg/mL or mg of sample	Easy, low-cost.
			qRT-PCR	Relative increase or reduction of expression	Special equipment is necessary. Standard and controls are necessary. Determine the gene expression but not the protein production.

		IL-10 has anti-inflammatory effect, and IL-1, IL-2, TNF-α or IFN-γ have pro-inflammatory functions.	Microarray	Relative increase or reduction of expression	Special equipment and software are necessary. Very accurate analysis. Expensive.
	Phagocytic activity	The activation of macrophages increases the clearance of invasive microorganisms and avoids the negative effects of bacterial translocation to internal organs. Measurement of natural immunity status.	Tissue culture	CFU/cell	It is necessary to have solid experience in primary cell culture. Time consuming.
	Cytolytic activity	Similar to phagocytic activity but measuring the reduction of the number of viable bacteria.	Tissue culture	% of CFU reduction	It is necessary a solid experience in primary cell culture. Time consuming.
	Lymphocyte proliferation	When B-cells are stimulated by their specific antigens, with the help of T-cells, B-cells are stimulated to undergo proliferation. When T cells are activated by antigen-presenting cells and cytokines, T-cells undergo proliferation.	Tissue culture	Lymphocyte count	It is necessary to have solid experience in primary cell culture. Time consuming.

Table 113: Summary of the principal end-points used for the efficacy evaluation of immunomodulatory substances / agents in fish and shellfish

	PARAMETERS	AIM/SIGNIFICANCE	METHODOLOGY	UNITS	COMMENTS
Performance					
	Body Weight Increase (BWI)	Basic performance data. In general, an increase of BWI is associated with positive effects of tested products.	Calibrated balance	Difference of individual body weight between different times, in kg	Easy collection. BW should be doubled or increased by three times in order to consider the trial reliable in terms of growth performance.
	Feed intake (FI)	Basic performance data. A reduction of FI associated to the maintenance of BW is associated with positive effect of tested products.	Calibrated balance	Weight of the feed consumed at different times, in kg	The individual measurement of feed intake is not possible under normal experimental conditions. Thus, the measure is collected for the animals into each experimental group. This value is a result of the feed administered and the feed not ingested, so the collection of uneaten feed is needed, an issue not always very evident.
	Feed Conversion Ratio (FCR)	Parameter that integrates BW and FI parameters. It is the principal indicator for animal performance.	Mathematical calculation	FI/BWI	The accuracy of this data depends on the accuracy of the feed intake measurement.
	Daily Weigh Gain (DWG)	An increase of DWG is associated with positive effect of tested products.	Mathematical calculation	BWI/days	Easy calculation. This parameter may be also represented as "specific growth rate, SGR" (% of BWI/day)
	Daily Feed Intake (DFI)	A reduction of DFI associated withthe maintenance of DBW is associated with positive effects of tested products.	Mathematical calculation	FI/days	Easy calculation.
	Carcass traits	Changes in the proximate composition (protein, lipid, carbohydrate, ash) of the carcass or fillet have been associated improved conditions and growth performance.	Biochemical analysis	%	Easy calculation. This parameter is generally a result of a better growth performance and its use has to be taken with caution.
Pathology					
	Clinical signs	Under field or experimental conditions, the evaluation of clinical signs is used for the	Direct observation	% of animals	Type and scale must be clearly defined in the experimental protocol.

		evaluation of the health status of animals.			
	Mortality	Primary indicator for the evaluation of the health status of animals.	Direct observation	% of animals	Easy collection. It is necessary to determine the causes.
Histopathology					
	Microvilli high	Gut health and digestive improvement is associated with an increase in microvilli high.	Staining and microscopy	µm (average of ten measurements per sample)	Easy collection but time consuming.
	Villi depth and width	An increase is associated with intestinal mucosa development.	Staining and microscopy	µm (average of ten measurements per sample)	Easy collection but time consuming.
	Intraepithelial lymphocytes	The persistence of inflammatory conditions at gut level is associated with the increase of intraepithelial lymphocytes.	Staining and microscopy	Number of cells (average of ten measurements per sample)	Easy collection but time consuming.
	Goblet cell	Increased goblet cells in the intestine (mucin-cells producing) are associated with improved condition of the intestinal mucosa	Staining and microscopy	Number and type (average of ten measurements per sample)	Easy collection but time consuming. Goblet cells may be stained with different dyes in order to decipher their content in neutral or acidic muco-substances. Histochemical procedures are available for lectin study in mucins.
Microbiology					
	Bacterial quantification	The quantification of some bacterial groups is used for the evaluation of the effects of probiotics and prebiotics on gut microbiota and gut health	Plate count	CFU/weight or volume of sample	Easy but time consuming, and not very specific as there a certain groups of bacteria that do not grow in plates.
	Study of bacterial population	The gut health is associated withspecific microbiota profiles. Dysbiosis is related tonegativeperformance and, in some cases, to digestive disorders.	DGGE or PCR-RFLP	Electrophoretic profiles	Easy but reduced specificity.
			Deep sequencing	OTUs quantification	The most accurate method. Special equipment and software are necessary. Expensive.
Immunology					
	Lysozyme activity	The quantification of the activity of lysozyme (mucolytic enzyme of leucocytic origin) provides information about the humoral non-specific immune response. This	Spectrophotometric assay	U/mL	Easy, low-cost.

		enzyme has antiviral, antibacterial, and anti-inflammatory properties.			
	Respiratory (oxidative) burst activity	The quantification of the activity of respiratory (oxidative) burst activity provides information about the cellular non-specific immune response, as neutrophils and monocytes release reactive oxygen species when they come into contact with different bacteria or fungi.	Spectrophotometric assay	U/mL	Easy, low-cost.
	Protease activity	The quantification of protease activity provides information about the cellular non-specific immune response.	Spectrophotometric assay	U/mL	Easy, low-cost.
	Immunoglobulins	The quantification of general or specific immunoglobulins is used for the evaluation of the improvement of humoral immune response by tested products.	ELISA	mg/mL	Easy, low-cost.
	Cytokine production	Small proteins are important tocell signalling during immune response. Some of theseare related tocellular immune response type 1 (IFN-γ, TNF-α), and others to cellular immune response type 2 (TGF-β, IL-4, IL-10, IL-13). IL-10 has anti-inflammatory effect, and IL-1, IL-2, TNF-α or IFN-γ have pro-inflammatory functions.	ELISA	µg/mL or mg of sample	Easy, low-cost.
			qRT-PCR	Relative increase or reduction of expression	Special equipment is necessary. Standard and controls are necessary. Determine the gene expression but not the protein production.
			Microarray	Relative increase or reduction of expression	Special equipment and software are necessary. Very accurate analysis. Expensive.
	Phagocytic activity	The activation of macrophages increases the clearance of invasive microorganisms and avoids the negative effect of bacterial translocation to internal organs. Measurement of natural immunity status.	Tissue culture - optical microscopy	The macrophage phagocytic activity (phagocytic index and percentage phagocytosis) of pronephros and spleen macrophages.	Not easy (cell separation). It is necessary to have solid experience in primary cell culture. Time consuming.
	Cytolytic activity	Similar to phagocytic activity but measuring the reduction of the number of viable bacteria.	Tissue culture	% of CFU reduction	It is necessary to have solid experience in primary cell culture. Time consuming.

	Complement activity	The quantification of complement activity (C3 and C4) is used for the evaluation of whether or not the classical complement pathway has been activated.	Tissue culture (sheep red blood cells)	% cell lysis	Easy, average-cost.
	Phenoloxidase activity	Phenoloxydase in haemolymph is one of the main defences of the organism functioning as a non-self recognition system. Primarily used in crustaceans (shrimp).	Spectrophotometry	U/mL	Easy, average-cost.
	Lymphocyte proliferation	When B cells are stimulated by their specific antigens, with the help of T-cells, B-cells are stimulated to undergo proliferation. When T-cells are activated by antigen-presenting cells and cytokines, T-cells undergo proliferation.	Tissue culture	Lymphocyte count	It is necessary to have solid experience in primary cell culture. Time consuming.
	Counts of different types of haemocytes (crustaceans)	Identification and quantification of different types of haemocytes (hyaline cells, semi-granular and large granular cells) according size and shape of the cells, presence of refractile granules in cytoplasm, characteristic staining of the nucleus and the intracytoplasmic content with Giemsa stain. Primarily used in crustaceans (shrimps).	Optical microscopy	Number of cells/mL	Easy, but experience and training in identifying different blood cells is necessary.
	Red and white blood cell counts (fish)	Identification and quantification of different cell types for leucocytes (eosinophil granulocytes, polymorphonuclear granulocyte, monocytes, lymphocyte).	Optical microscopy	Number of cells/mL % red blood cells % white blood cells	Easy, but experience and training in identifying different blood cells is necessary.
			Blood counter by centrifugation	Haematocrit	Easy, average-cost.

3.7. Risks for the safety of target animals, consumers, users, and the environment

The questionnaire (DistillerSR) performed for each study included in the systematic research had a specific section in which the safety for target animals, consumers, users, and the environment was assessed. A reduced number of studies evaluated the safety of the immunodulatory substance / agent. This might be explained by the main purpose of the tender being identification of substances / agents with immunomodulatory effects likely to be used as feed additives (mode of action and end-points) rather than the safety of these substances. A chain of searches with specific key words for each immunomodulatory substance /agent identified should be performed in order to evaluate safety.

3.7.1. Risks for the safety of target animals

The aim of the studies focusing on the safety for target animals is to provide a limited evaluation of short-term toxicity of the additive used in the feed of target animals. This is also used in order to establish a margin of safety if the additive were consumed at a higher dose than is recommended (tolerance)(EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011). Due to the potential immunodulatory activity of these substances / agents, tolerance studies should be required because the metabolic activity of these substances/agents, particularly in cases where safety checks have not been performed (perhaps because substances have not yet been considered as feed additives yet).

In 2007, the EFSA's Scientific Committee established the first list of proposed biological agents for the Qualified Presumption of Safety (QPS)(EFSA Scientific Committee, 2007). This list is regularly updated by the EFSA Panel on Biological Hazards (BIOHAZ). The last amendment is on 2015 (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2015). The list includes the following categories of microorganisms: gram (+) non-sporulating bacteria, gram (+) spore forming bacteria, gram (-) bacteria, yeast, filamentous fungi, oomycetes, and viruses used for plant protection. The safety of the microorganisms that are not included in this list should be evaluated.

Common prebiotics in use include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins and isomalto-oligosaccharides. These saccharides have a long history of safe use and are generally regarded as safe although there is some concern over increased gas production with some compounds, particularly when ingested in higher amounts or during the first few days of intake. There is also a range of new prebiotic compounds emerging, including: pecticoligosaccharides, lactosucrose, sugar alcohols, gluco-oligosaccharides, levans, resistant starch, xylosaccharides and soy-oligosaccharides. These compounds have been studied to varying degrees in vitro and in animal feeding studies but rarely in human feeding studies (Pineiro et al., 2008).

Botanicals contain hundreds of species / subspecies, few of which have been fully characterised; consequently, any pre-assessment may be limited to a single species or even an extract or a specific part of a plant. In addition, morphology and chemical composition of plants may be markedly affected by geographical and environmental factors, not least from the selection of cultivars appropriate for a given region. All of these factors will influence the possibilities for the grouping of the botanicals and for botanical preparations in a QPS approach; the factors will also influence the decision as to what materials can be included in the assessment. The lack of category-specific data and potential differences in pharmaco-kinetics found amongst livestock, including fish, means that it is unlikely that a composite conclusion necessary for a QPS approach could be achieved (EFSA Scientific Committee, 2014).

The potential application of immunomodulatory animal by-products must take into consideration Article 11 of Regulation (EC) 1069/2009 (Regulation, 2009), which details restrictions on use relating to feeding of animal by-products and bans intra-species recycling (feeding material derived from a species to a creature of the same species) as well as the feeding of catering waste to farmed animals.

The list of substances /agents included in the category of "other substances" includes a wide range. Some of these are already feed additives approved by EU regulations (i.e. vitamins, amino acids, minerals). Therefore, the safety of the target animals has already been assessed. However, other *algae*, fungi, nucleotides, and organic acid derivatives are complex groups of substances for which safety needs to be assessed on a case-by-case basis.

3.7.2. Risks for the safety of consumers

The assessment of the safety of the use of additives is related to consumer exposure to food products derived from animals given feed or water containing or treated with the additive and containing residues of the additive or its metabolites. If these substances / agents are not absorbed by the animal, an assessment of the safety of consumer is not required.

3.7.3. Risks for the safety of users

Users / workers are defined as the individuals who may be exposed to an additive while handling it, when incorporating it into pre-mixtures or feeding-stuffs, or when supplementing feed with the additive.

3.7.4. Risks for the safety of the environment

The assessment of the environmental impact of additives is important as administration of additives typically occurs over long periods of time, involve large groups of animals, and the constitutive active substance(s) may be excreted to a considerable extent either as the parent compound or its metabolites.

In order to determine the environmental impact of additives, a step-wise approach ought to be followed. All additives must be assessed through Phase I in order to identify additives which do not need further testing. Natural and physiological non-toxic substances, such as vitamins, carotenoids, proteins, biodegradable compounds and substances with low content below Predicted Environmental Concentration (PEC, according to EFSA guidance EFSA –Q-2008-408 (EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2008)) according to aquatic and terrestrial environments, are considered generally safe for the environment. For the other additives, a second phase (Phase II) of assessment is required in orderto provide additional information, based on which further studies (such as Eco-toxicity tests in terrestrial, freshwater and marine compartments, as well as Biodegradability assays) may become necessary. These studies shall be conducted according to Council Directive 67/548/EEC.1 (Regulation, 2008b).

3.7.4.1. Safety for the environment with regard to probiotics

Probiotics feed additives are live microorganisms (bacteria and yeasts) than, when ingested, have a positive effect on the health and nutritional status of livestock and humans. Microorganisms used in animal feed in the EU are mainly bacterial strains of gram (+) bacteria belonging to the types *Bacillus*, Lactic acid bacteria such *as Enterococcus, Lactobacillus, Pediococcus, Lactococcus, Streptococcus, Pediococcus, Bifidobacterium,* and strains of yeast belonging to the *Saccharomyces cerevisiae* and *Kluyveromyces* (Anadón, Rosa Martínez-Larrañaga, & Aranzazu Martínez, 2006; Salminen et al., 1998; Y.-B. Wang, Li, & Lin, 2008).

Lactic acid bacteria (LAB) in foods have a long history of safe use. Members of the genera Lactococcus and Lactobacillus are most commonly given generally-recognised-as-safe (GRAS) status whilst members of the genera *Streptococcus* and *Enterococcus*, and some other genera of LAB, contain some opportunistic pathogens (Salminen et al., 1998). Although lactic acid bacteria are intrinsically resistant to certain antibiotics, in many cases antibiotic resistance is not transmissible, and this will not necessarily translate into sensitivity to clinically used antibiotics (Lim, Huh, & Baek, 1993; Pan & Yu, 2014; Salim Ammor, Belen Florez, & Mayo, 2007; Sherman, Ossa, & Johnson-Henry, 2009). Therefore, in cases of probiotic occurrence in manure / excretion products, no particular safety concern is associated with this intrinsic type of resistance.

In the case of spore formers, such as *Bacillus cereus* and enterotoxins, emetic toxin must be monitored in manure / excretion products (Ripamonti & Stella, 2009; Sanders, Morelli, & Tompkins, 2003). In addition, potential synthesis of mycotoxins and toxins produced has been described in *Clostridium* and *Aspergillus* (Sabino, Faisca, Carolino, Verissimo, & Viegas, 2012), and should also be assessed by means of molecular-based detection kits in the case of probiotic presence in excreted products and manure (EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2014).

The environmental risk assessment would also be required in cases of additive persistence in manure / excretion products containing Genetic Modified Organisms (GMOs) within the meaning of article 2 (1) and 2 (2) of Council Directive 2001/18/EC (Directive, 2001). This should satisfy the requirements of the release directive, which includes an assessment of any

potential risks to the environment which are related to the GMOs (Anadón, Roda, & Martínez-Larrañaga, 2004).

3.7.4.2. Safety for the environment with regard to prebiotics

Naturally, non-digestible but fermentable carbohydrates such as mannan oligosaccharide (MOS), glucans, fructooligosacharides (FOS), the yeast cell wall (YCW), inulin, chitooligosacharides (COS), and galactooligosacharides (GOS) are utilised as prebiotic additives in order to stimulate fermentative metabolism of anaerobic bacteria by generating volatile fatty acids (VFA) as end products (Y. Wang, 2009). VFA occurrence (biogenic origin) may promote microbial growth in the gut and in natural environments in case of excretion (Sghir, Chow, & Mackie, 1998). According to technical guidance for assessing the safety of feed additives for the environment (EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2008), prebiotics can be considered safe for the environment in Phase I due to its natural origin, high biodegradability, and low Eco-toxicity (Sousa, dos Santos, & Sgarbieri, 2011). Therefore, no additional scientific research is needed with regard tothis point at this juncture.

3.7.4.3. Safety for the environment with regard to plant extracts

Herbs, spices, and their extracts have a wide range of activities. They can stimulate feed intake and endogenous secretions or have antimicrobial, coccidiostatic, or anthelmintic activity as well as protect animals and their products from oxidation (Wenk, 2003). Plant extracts are composed of a wide range of low-molecular-weight secondary metabolites, which belong to the classes of isoprene derivatives, flavonoids, glucosinolates, essential oils, and dietary fibre (Halliwell, Aeschbach, Loliger, & Aruoma, 1995; Hirasa & Takemasa, 1998; Rhodes, 1996). Knowledge of the effects of secondary plant metabolites in feed and guts, their bioavailability by means of extent of absorption, metabolism and excretion, and the extent to which they might be retained in animal tissues is not readily available and ought to be further investigated (Wenk, 2003). According to technical guidance for assessing the safety of feed additives with regard to the environment (EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2008), plant extracts could be considered safe for the environment in Phase I (EFSA-Q-2008-408) due to its natural origin and high degree of biodegradability. However, due to complex and sometimes scarcely

known composition, additional toxicity assays should be requested in order to gain insight in to the environmental fate of secondary plant metabolites and their transformation products that could occur in manure and excretion products.

3.7.4.4. Safety for the environment with regard to animal by-products

Animal by-products enriched in protein content, such as lactoferrin, spray-dried plasma and antibodies, are natural and physiological non-toxic substances that are typically given in low dosages; in case of their occurrence in manure and excretion products, they are easily biodegradable in a soil and water system environment. Therefore, according to technical guidance for assessing the safety of feed additives for the environment (EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2008), they can be considered safe for the environment in Phase I due to its natural origin and high degree of biodegradability.

3.7.4.5. Safety for the environment with regard to other feed additives of interest

In addition, other organic feed additives naturally present (such as organic acids, vitamins, amino acids and derivatives, *algae*, enzymes and fungi) at low dosages and in the absence of toxins and pesticides (*algae*) can be considered safe for the environment. In the case of mineral radioactivity (Lieser, 1995) heavy metal composition must be assessed during additive evaluation.

3.8. Existing legislation on the authorisation of substances / agents identified in the tender of third countries

All safety agencies in third countries provided by EFSA were contacted, but only two answers were received. Both agencies had a similar reaction to the term "immunostimulator". They do not consider all of these substances / agents to be feed additives but rather consider them to have a direct effect on the immune system, as a veterinary drug. No response was received from any of the other institutions that were contacted.

3.9. List of patents for substances / agents identified in the tender

The results of the specific patent search (using Questel-Orbit© software) of each product obtained from the systematic review are available upon request.

In the following tables, the number of patents for each of the substances / agents for probiotics (Table 114), prebiotics (Table 115), plant extract (Table 116), animal by-products (Table 117), and other substances (Table 118) are listed.

Table 114: Number of patents for each probiotic included in the systematic review

Probiotic	Number of patents
Aeromonas spp.	28
Alteromonadaceae spp.	0
Arthrobacter spp.	8
Aspergillus spp.	91
Bacillus spp.	595
Bifidobacterium spp.	103
Candida spp.	52
Carnobacterium spp.	5
Citrobacter spp.	8
Clostridium spp.	76
Debaryomyces spp.	0
Dietzia spp.	0
Enterobacter spp.	5
Enterococcus spp.	83
Escherichia spp.	113
Flavobacterium spp.	1
Hafnia spp.	5
Halomonas sp	0
Hanseniaspora spp.	0
Lactobacillus sp	410
Lactococcus spp.	41
Leucobacter spp.	0
Leuconostoc spp.	27
Luteimonas spp.	0
Lysinibacillus spp.	1
Megasphaera spp.	3
Methylococcus spp.	0
Microbacterium spp.	1
Micrococcus sp	21

Mucor	12
Paffia spp.	0
Pantoea spp.	6
Pediococcus spp.	40
Phaeobacter spp.	0
Plesiomonas spp.	0
Propionibacterium spp.	21
Pseudomonas spp.	39
Psychrobacter spp.	0
Rhodococcus spp.	0
Rhodopseudomonas spp.	1
Saccharomyces spp.	180
Shewanella spp.	3
Sphingopyxis spp.	0
Staphylococcus spp.	48
Streptococcus spp.	89
Streptomyces	32
Vagococcus spp.	1
Vibrio spp.	54
Weixella spp.	0
Zooshikella spp.	0
TOTAL	**2203**

Table 115: Number of patents for each prebiotic included in the systematic review

Prebiotic	Number of patents
Acidic oligosaccharides (AOS)	0
Arabinogalactan	2
Arabinoxylan / Arabinoxylan oligosaccharides (AXOS)	7
Chitin	50
Chitooligosaccharide (COS)	5
Fructooligosaccharide (FOS)	21
Galactomannan / Galactomannan oligosaccharides (GMOS) /Galactoglucomannan oligosaccharide-arabinoxylan complex (GGMO-AX)	7
Galactooligosaccharides (GOS)	8
Glucan	113
Inactivated bacteria	4
Inactivated yeast	2
Inulin	27
Levan	3
Lipopolysaccharide (LPS)	35

Mannan oligosaccharide (MOS) / Phosphorylated mannans (MAN)	62
Mannobiose	1
Peptidoglycan	21
Polydextrose	5
Transgalactooligosaccharide (TOS)	0
Xylooligosaccharide (XOS)	12
Yeast cell wall (YWC)	48
TOTAL	**433**

Table 116: Number of patents for each plant extract included in the systematic review

Plant extract	Number of patents
Acacia derivatives	18
Achillea	6
Achyranthes derivatives	21
Aconitum koreanum	0
Agrimonia	19
Allium derivatives	9
Alnus firma	2
Anethole	2
Artemisia derivatives	80
Arthropodium cirratum	0
Astragalus derivatives	1
Avena sativa	44
Azadirachta indica	5
Beta vulgaris	1
Bran derivatives	1807
Broussonetia kazinoki	4
Camellia sinensis	50
Canavalia ensiformis	1
Capsicum derivatives	14
Carotenoids	68
Carthamus	101
Carvacrol	7
Ceratonia siliqua locust bean	9
Chicory	42
Cinnamon derivatives	74
Citrus by product	513
Cocos nucifera	47
Coriandrum Sativum	10
Cucurbita	7
Cuminum cyminum	0

Curcuma longa derivatives	17
Cyamopsis derivatives	30
Echinacea	45
Eleutherococcus senticosus	12
Epimedium derivatives	71
Foeniculum vulgare	62
Garcinia mangostana	0
Ginkgo biloba	0
Glycyrrhiza	59
Gossypium	101
Helianthus tuberosus	0
Houttuynia cordata	57
Humulus	49
Ilex paraguarensis	10
Isoflavones	34
Juniperus communis	3
Kalopanax pictus	0
Lactuca indica	28
Larix	0
Laurus nobilis	4
Linum usitatissimum	15
Lonicera	246
Lupinus perennis	6
Malus domestica	61
Mangifera indica	32
Medicago sativa	282
Mentha piperita	2
Myrtus communis	2
Nigella sativa derivatives	0
Origanum derivatives	16
Oscimum sanctum	1
Other Chinese herbs	696
Panax ginseng	271
Phoenix dactylifera	97
Pimpinella anisum	30
Pinus derivatives	11
Piper nigrum	80
Pisum sativum	19
Plantago derivatives	6
Propolis	52
Prunus derivatives	10
Quillaja saponaria	0

Raw fibre	1
Rosmarinus officinalis	22
Rubus coreanus	2
Sanguinaria canadensis	0
Silybum marianum	11
Solanum tuberosum	521
Soybean derivatives	1246
Sugar cane	86
Syzygium aromaticum	103
Tannin	23
Thymus vulgaris derivatives	24
Trigonella derivatives	11
Uncaria tomentosa	2
Urtica dioica	23
Viscum album	0
Withania somnifera	2
Yucca Schidigera	16
Zea mays	1627
Zingiber officinale	270
TOTAL	**9469**

Table 117: Number of patents for each animal by-product included in the systematic review

Animal by-product	Number of patents
Antibodies	332
Fish oil	219
Lactoferrin	24
Poultry oil	0
SDP (spray-dried plasma)	4
TOTAL	**575**

Table 118: Number of patents for each other substance / agent included in the systematic review

Other	Number of patents
Algae	264
Amino acids and derivatives	77
Enzymes	963
Fatty acids	267
Fungi - Mushroom	375
Lactulose	11
Minerals	981
Nucleotides	220

Organic acids derivatives	93
Peptides	459
Vitamins	2219
TOTAL	**5929**

4. Discussion

The word "stimulate" is used differently by immunologist and pathologists than it is for nutritionists. In order to avoid confusion, Klasing (2007) suggested that the word should be used only in its immunological context, that is, something that causes the immune system to respond (Klasing, 2007). Thus, pathogens, lectins, and irritants all stimulate the immune system. Microbial and food antigens engage the immune system and induce differentiation of lymphocytes during development. Diet can nourish immune cells, modulating them and facilitating the establishment of commensal microflora. However, diet should not normally stimulate the immune system.

Due to the emergence of microbes resistant to antibiotics used to treat human and animal infections, the European Commission (EC) has decided to phase out, and ultimately ban (January 1st 2006), the marketing and use of antibiotics as growth promoters (AGP) in animal feed (Regulation, 2003).

Alternatives to growth promoters should carry the same beneficial effects as AGPs. However, the manner in which AGPs exert their beneficial action is not entirely known. The most well-known mechanism to be proposed thus far is the idea that AGPs have an antibacterial action that favours performance in different ways: (1) by reducing the incidence and severity of subclinical infections (Brennan, Skinner, Barnum, & Wilson, 2003; George, Quarles, & Fagerberg, 1982); (2) by reducing the microbial use of nutrients (Snyder & Wostmann, 1987); (3) by improving the absorption of nutrients due to the thinning of the intestinal wall, (4) by reducing the amount of growth-depressing metabolites produced by Gram (+) bacteria (Feighner & Dashkevicz, 1987; Knarreborg, Lauridsen, Engberg, & Jensen, 2004) and (5) by inhibiting inflammatory response (Niewold, 2007).

The focus of alternative strategies (i.e. the addition of immunomodulatory substances / agents in animal diets) has been the prevention of the proliferation of pathogenic bacteria and the modulation of commensal bacteria, the modulation of local and systemic immune response, and the improvement of welfare and health status.

4.1. Porcine

The most common additives studied with regard to immunity in swine were probiotics. Among these 186 studies, the majority were centred on *Lactobacillus* (69 studies), *Bacillus* (31 studies), *Enterococcus* (29 studies), and *Saccharomyces* (30 studies).

In swine, two main mechanisms of action have been described for *Lactobacilli*. They prevent the proliferation of pathogenic microorganisms primarily through the reduction of pH and the production of lactic acid and VFA. They also have a direct action on the immune system through interacting with intestinal TLR and NOD as well asactivation of NLRP3. *Lactobacillus* treatment increased intra-epithelial lymphocytes and IgA-producing cells in the intestinal tract and increased peripheral blood CD4+ T-lymphocytes. The same experiment also yielded an anti-inflammatory effect of *Lactobacillus,* with a decreased expression of mRNA encoding for pro-inflammatory cytokines such as Il-1β, Il-8, Il-17 and TNF-α and an increased production of immunoregulatory factors, such as TGF-β and IL-10.

In swine, *Bacillus ssp*. triggers the immune response modulating the lymphocyte populations and increasing the local and systemic concentration of antibodies. They also decrease *E. coli*-induced inflammation by acting on the MAPK signaling pathways. *Bacillus* strains may also alter the type of microflora in the gastrointestinal tract through: (i) decreased oxidation–reduction potential caused by the germination of spores in the intestine, which has been shown to benefit the growth of *Lactobacilli*, and (ii) through the production of some antimicrobials, such as aminocumaim A, bacteriocin, and defensins.

Saccharomyces cerevisiae does not change the gut architecture, mucus thickness, goblet cell population, or cortisol level but is able to promote the growth of *Lactobacilli*. *Saccharomyces* administration increases the serum levels of antibodies, gamma-globulins, lysozyme, and IFN-γ, IL1β and IL-6 levels. It also increases the proportions of CD4+, CD8+ and CD4+CD8+ peripheral blood lymphocytes, suggesting an activation of systemic cell-mediated immunity. Live yeast reduced the attachment of *E. coli* to the ileal mucosa through various mechanisms, such as modulation of the TLR receptor as well as competition, modulation of intestinal bacterial populations, and increased barrier function. *Saccharomyces* also down-regulate *E. coli*-induced inflammation, as measured by several inflammatory cytokines; this

inhibition was associated with the modulation of MAPKinase signaling pathways, the increase of PPAR-c transcript expression, and bacterial agglutination.

Compounds of *E. faecium* EF1 cell-wall, like DNA, could act as adjuvants of the mucosal immune response. Studies also suggest that *E. faecium* suppresses the synthesis of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, IL-8 and IFN-γ) and increases the synthesis of anti-inflammatory cytokines (IL-10, TGF-β). Application of *E. faecium* affects the systemic and intestinal immunity of weaning piglets by inducing the proliferation of CD4-CD8 cells, thus decreasing the proportion of circulating and intraepithelial CD8αβ T-cell and cytotoxic T-cells (CD8+). A reduction in β-haemolytic *E. coli* isolates in probiotic-treated animals suggests a reduced pathogenic load in the probiotic group.

Sixty-four studies explore the effects of prebiotics. The most commonly studied prebiotics were mannan oligosaccharides (MOS, 12 studies), glucans (nine studies), fructooligosaccharides (FOS, four studies), inulin (source of FOS, seven studies), the yeast cell wall (source of MOS amongst other substances, six studies) and chitooligosaccharide (COS, six studies).

In both control pigs and those infected with PRRS, MOS reduced the expression of inflammatory cytokines (IL-1, IL-8, TNF, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1). MOS also modulated TLR4 and TLR5 expression, increased IgG concentration, and tended to increase the serum concentration of IL-10. It did not affect the TNF-α. MOS decreased jejunal numbers of *Enterobacteria* and increase the villus:crypt ratio.

β-Glucan did not alter final BW, ADG, or G:F and did not impact villus height or crypt depth. Piglets receiving food supplemented with β-glucans after weaning show a decreased susceptibility to ETEC. β-Glucan down-regulated the expression of pro- and anti-inflammatory cytokines (IL-1α, IL-10, TNF-α and IL-17A) in the colon. It also decreased plasma level of IFN-γ and TNF-α levels while increasing the one of IL-10. Oral supplementation with β-1,3/1,6-glucan resulted in temporarily increased serum IgG concentrations of weanling piglets. Supplementation of dietary β-glucan resulted in an increased concentrations of peripheral erythrocytes, leukocytes, lymphocytes, and eosinophils as well an increased proportion of CD45RA+ T-cell populations (mesenteric

lymph nodes and Peyer's patch CD4+ cells, and PBL CD8+ cells) were greater in pigs fed β-glucan.

The overall performance of piglets was unaffected by inulin treatment. Inulin increased total aerobes in the stomach and jejunum while decreasing enterococci in the colon. Furthermore, decreased colonic acetic acid and increased lactic acid was observed. Inulin may contribute to the regulation of Fe absorption, either directly by affecting SCFA synthesis or through changes in bacterial population and/or by affecting mucin gene expression. Inulin is able to increase the level of cytokine (IL-1β, IL-8, IL-6, TNF-α, IFN-γ) and IgA concentration.

Morphologically, increased villous density was observed in the duodenum of piglets fed diet with FOS.

A substantial number of studies (68) concern plant extracts. With the exception of soybean derivatives, the mode of action of these plant extracts has been poorly investigated either because they were primarily studied in mixtures (carvacol or Chinese herbs) or because few studies are available (*Thymus, Astragalus*, *Capsicum*, *Curcuma longa* or *Achyranthes* derivatives, *Echinacea*, *Glycyrrhiza*, sugar cane or bran derivatives.

Several soybean derivatives have been tested in pigs. Soybean oligosaccharides promoted the growth of bacterial species that are potentially beneficial to the host and increased the concentration of SCFA in the ileum and colon while simultaneously reducing the number of potentially harmful bacteria and the production of protein-derived catabolites. They increase the expression of ZO-1 mRNA but diminished the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-8 mRNA and increased that of the anti-inflammatory cytokine IL-10.

Pigs treated with soy peptides showed reduced expression of inflammatory cytokines (IFN-γ, TNF, and IL12B) as well as genes involved in differentiation of Th17 cells (IL17A and RORC) at the expense of the pathway of regulatory T-cells (FoxP3). Soy di-and tripeptide decreased crypt depth.

Antigenic soybean product increased diarrhoea, reduced the size of villi and eosinophil density in the duodenum more so than the non-antigenic soybean product. Beta-sitosterol (phytosterol of soybean) increased peripheral blood mononuclear cell numbers and activated

swine dendritic cells. Heat-treated soy protein had more of an effect than ethanol-treated soy protein. It increases the cell density in the villus Lamina Propia as well as the number of CD4+ T-cells, IgGl and IgG2 positive cells. This was associated with an increased level of IgM and IgA.

Twenty-eight studies concern animal by-products, primarily spray-dried plasma (11 studies). Antibodies from egg-yolk or colostrum (seven studies), lactoferrin (five studies), and fish oil (two studies) were also tested.

The effect of SDP on the performance is unclear at the time of writing. Piglets fed with an SDPP diet showed increased levels of IgG and reduced numbers of blood monocytes. In the intestine, SDP reduced the percentage of macrophages (SWC3+), B lymphocytes (CD21+), γδ+ T cells (γδTCR+) in LN, and intraepithelial lymphocytes. It also increased duodenal villus height and reduced malondialdehyde content in mucosa. In the mucosa, SDP decreased TNF-α, IL-6, IL-1β, TGF-β and soluble IL-2 receptor content; it did not alter the cytokines in the serum.

Other substances were investigated across 58 studies. These mainly concerned organic acid derivatives and fatty acid (14 studies), minerals (11 studies), and amino acids and their derivatives (19 studies).

4.2. Poultry

The different bacterial species in the normal microbiota (colonising on the epithelium of the digestive tract or occurring freely in the gut lumen) of the broiler gut reach a typical equilibrium state approximately a week after hatching; this is dependent on many factors, including location in the gastro-intestinal tract, integrity of the intestinal mucosa, and transit time of the chymus (Teirlynck et al., 2009; van Der Wielen et al., 2000).

Depending on the probiotic strain, the mode of action likely involves the production of specific metabolites (short organic fatty acids, H_2O_2, intermediary metabolites with antimicrobial activity), interaction with receptor sites, stimulation / modulation of the

immune system, and several others (Huyghebaert et al., 2011; Madsen, 2001; Sherman et al., 2009). The most well known group of probiotics are lactic acid bacteria (in particular, *Lactobacillus*; 113 articles).

The administration of live non-pathogenic bacteria in poultry diets has the aim of (1) displacing pathogenic microorganisms for competitive exclusion colonising the gut tract, (2) avoiding growth and adhesion / translocation / colonisation of pathogenic microorganisms, and (3) modulating the innate immune system. The primary aim of immunomodulatory feed additives is the reduction of local inflammation and limitation of further impairment of immune function.

The early post-hatch period is a critical stage for poultry growth and health.The rapidly developed intestinal tract, at this stage of growth, provides an ideal niche for microbial colonisation. In the meantime, gut microbiome also plays an important role in intestinal development (Pan & Yu, 2014).

As a component of the intestinal mucosal innate immune system, the mucus layer prevents gut microorganisms from penetrating into the intestinal epithelium, serving as the first line of defence against infection. Other important components of the innate immune system functioning in the avian gut are the antimicrobial peptides present on the intestinal epithelial surface (i.e. β-defensins). Intestinal morphology can be affected by dietary supplementation of different immunomodulatory substances/agents, increasing villus height in the duodenum and the villus height:crypt depth ratio in the ileum of broilers.

Cellular components of the avian innate immune system, such as macrophages and heterophils, also protect the host from enteric infection. These cells can be found in peripheral circulation and the lamina propria. When intestinal microorganisms breach the intestinal epithelial barrier, these immune cells are recruited to the site of infection, where they kill the invaders using a variety of strategies, such as phagocytosis and oxidative burst (Brisbin, Gong, & Sharif, 2008).

Toll-like receptors (TLR) are germ line encoded pattern recognition receptors that recognise conserved structural motifs known as pathogen-associate molecular patterns expressed by microbes (Akira, Takeda, & Kaisho, 2001). After TLR activation, one possible outcome is the

synthesis and release of pro-inflammatory cytokines. The presence of these cytokines modulates adaptive immunity. B- and T-cells, which elicit antibody-mediated and cell-mediated immune responses respectively, are the two primary types of lymphocytes of fundamental importance to the adaptive immune system. In the avian gut, B- and T-cells can be found in organised lymphoid tissues (e.g. caecal tonsils, Peyer's patches, thymus, and the bursa of Fabricius) as well as in more dispersed areas, such as the lamina propria and the epithelium (Brisbin et al., 2008; Haghighi et al., 2006). Studies have indicated that manipulation of the gut microbiome, through administration of immunomodulatory substances / agents, can influence cell- and antibody-mediated immune response (Pan & Yu, 2014).

The first two weeks post-hatch are of significant immunological importance as the chick is immediately exposed to environmental antigens and pathogens, resulting in changes to intestinal lymphocytes and the temporal development of lymphocyte functions (Bar-Shira, Sklan, & Friedman, 2003). Therefore, studies exploring such effects on the immune system for immunomodulatory substances, in cases of poultry, should be performed during the first weeks of life (not later than 21 days). Those experiments should analyse microbiota, microvilli, the mucosa structure, innate immunity (TLR up/down regulation), differentiation of naïve T-cells into mature effector cell types (Th1, Th2, Th17 and Treg functions), and several types of cytokine expression.

Additionally, it would be interesting to associate the results obtained from early immune response with the performance of the animal at the end of the process of the fattening period as applicable to chickens.

4.3. Bovine, ovine, and caprine

For both large and small ruminants, the feed additives with the most published research studies are probiotics (mainly bacteria). Within this group, Lactic acid bacteria (LAB, such as *L. acidophilus*, *L. plantarum, L. paracasei,* and *L. animalis,* among others) have been shown to improve both BW gain and feed intake. In some instances, LAB have been shown to reduce the number of *E. coli* strains isolated from the gastrointestinal tract of cattle. In a

similar vein, some studies have shown that LAB have effectively reduced the number of microorganisms capable of causing inflammation due to the production of organic acids (acetic and lactic acid), hydrogen peroxide, and bacteriocins. More specifically, some LAB, under certain conditions, may improve the expression of IL-4R and have potential to increase IL-1b production and enhance cell death. A possible mechanism by which the additive increases neutrophil IL-1β secretion is via IL-4R-dependent activation of the IL-1β converting enzyme (ICE). Increased expression of the EGF receptor implies that neutrophils of the treated animals may be more sensitive to EGF signaling and therefore may be more sensitive to TNF.

Other bacterial probiotics that have been studied extensively are *Enterococcus* and *Bacillus*; however, the modes of action of these bacteria are less consistent and less well-described. The literature reports primarily overall improvements in health (such as the reduction of diarrhoea, morbidity, and death).

There are many more bacterial probiotics used in ruminants, but the literature about these bacteria is scarce.

Saccharomyces spp. is a yeast probiotic that has been reported to exert influence effects on the health of young ruminants. However, their mode of action is not very well understood / described at this point in time.

There is only one study using fungal probiotics (*Aspergillus spp.*), but its results were inconclusive.

The literature around probiotics is also limited. The most consistent effect of these substances on immune function, in both large and small ruminants, has been reported for mannan oligosaccharides; again, the mode of action here has not been well identified at the time of writing.

A range of plant extracts has been tested in ruminants. However, as commonly occurs with these products, there is significant variability in the results (partly due to the inherent variability within the raw material), and the studies provide only speculation about potential mode of actions rather than reaching a clear conclusion.

The potential effects of trace minerals on immune function have been moderately reported in the literature, yet no clear mode of action has been proposed, and the majority of studies report only overall health improvements and speculate about potential underlying mechanisms behind these outcomes.

There are many other feed additives (sodium butyrate, lactoferrin, algae, nucleotides, etc.) for which there is only one study and no clear conclusion could be derived.

4.4. Fish

4.4.1. Salmonids

4.4.1.1 Salmonids – probiotics

Lactobacillus spp., *Lactoccocus spp*. and *Bacillus spp*. are probiotic bacteria that have primarily been tested in salmonid species, representing 41% of the scientific articles published in this area. The analysis of the mode of action of different probiotics in salmonids indicated that, between the 26 different probiotic bacteria tested, eight showed *Lactobacillus spp., Lactococcus spp., Bacillus spp., Enterococcus spp., Carnobacterium spp., Pediococcus spp., Pseudomonas spp., Methylcoccus spp.*, probiotic bacteria as competing with pathogenic bacteria in their adherence to the mucus layer, covering the intestinal epithelium, interfering with pathogen colonisation in the gut and in most cases modulating the host immune response. In addition, the above-mentioned competition with a potential pathogen in the colonisation of the gut may result in changes to the microbiota and in the density of intestinal goblet cells, as has been reported with *Bacillus* spp. This analysis does not imply that this mode of action is not present in the remainder of the probiotics tested in salmonids, as there has been a lack of coherence in the methodological approach for assessing the mode of action of different feed additives and, consequently, this parameter was not assessed in scientific studies exploring the remainder of the probiotic bacteria listed in Table 31.

The aforementioned increase in the density of intestinal goblet cells producing the layer of mucus covering the intestinal epithelium has been indirectly associated with the

enhancement of immune function of the intestine; studies conducted with *Bacillus spp*. and *Pantoea spp*. indicate this, although further validation with specific studies and challenges with pathogenic bacteria is necessary.

Regarding the enhancement of the immune response in salmonids as a consequence of dietary probiotic administration, different data is available concerning the set of biological, biochemical, and molecular set of markers selectedby different authors. For instance, *Lactobacillus spp*. and *Aeromonas spp*. have been reported to enhance the non-specific humoral defence (lysozyme, complement activation and bactericidal activity), whereas *Lactobacillus spp*. also increased the expression of a pro-inflammatory cytokine (IL-1β1) and TGF-β genes in the spleen and kidney. Similar results were reported for *Enterococcus spp*., which increased the levels of expression of the IL-1β1 gene. In the case of *Bacillus spp*., the administration of *B. subtilis* strain AB1 and *Micrococcus spp*. have been reported to increase both cellular and humoral (lysozyme, phagocytic activity and respiratory burst activity) immune responses.

In some other studies, *Lactoccocus spp*. has been described to enhance the phagocytic activity of the head kidney leucocytes, *Vibrio spp*., the number of erythrocytes, kidney macrophytes, the proportion of lymphocytes to monocytes, *Pseudomonas spp*., the number of leucocytes, IgM production, and serum lysozyme levels. In addition, some probiotic bacteria (e.g. *Aeromonas spp*., *Bacillus spp*.) have been reported to increase the production of chitinase and siderophores (iron chelating compound limiting bacterial growth).

The administration of the yeast *Saccharomyces spp*. had similar effects to probiotic bacteria by enhancing humoral (lysozyme, complement activity, alternative complement) and cellular (neutrophil number) immune responses.

In some of the literature analysed, some probiotics (e.g. *Leuconostoc spp., Luteimonas spp., Rhodococcus spp., Microbacterium spp., Sphingopyxis spp., Leucobacter spp., Diezia spp*.) were tested within a mixture of different probiotic bacteria and, consequently, it was impossible to decipher their specific mode of action. Other probiotic bacteria, such as *Plesiomonas spp., Hafnia spp., Enterobacter spp., Citrobacter spp., Lysinibacillus spp.* and *Staphylococcus spp*., have been identified as candidates for the control of the bacterial cold water disease in salmonids; however, their mode of action was not described.

4.4.1.2 Salmonids – prebiotics

It is important to mention that the amount of literature exploring probiotics in salmonids was 4.3 times higher than literature about prebiotics. The number of different additives tested was also higher here. Due to their different origins, characteristics, and properties, prebiotics exert different effects on the organism; unlike with probiotics, it is not simple to generalise their modes of action. Among the different prebiotics tested in salmonids, β-glucan was the most frequently evaluated in this group of fish (50% of revised manuscripts dealing with prebiotics focused on β-glucans). β-glucan enhances the innate immune factors by up-regulating expression of complement factors (C3 and factor B) and acute phase proteins (hepcidin, precerebellin and transferrin); in addition, β-glucan up-regulated the expression of the MHC-IIa chain, suggesting a role for adaptive immunity after dietary immunostimulation in fish as well as influencing the specific response to an antigen in terms of antibody response to vaccination and lymphocyte proliferation. Mannan oligosaccharides (MOS) and fructooligosaccharides (FOS) were two of the most frequently tested prebiotics in salmonids. MOS was reported to modulate intestinal microbial communities, which subsequently improved gut morphology and the epithelial brush border. Additionally, MOS up-regulated the lysozyme and TNF-α and down-regulated HSP70 gene expression. It has been also suggested that MOS stimulates mannose binding lectin (MBL) through liver secretion binding the capsule of bacteria and triggering the complement cascade. Regarding MOS, as these compounds have been tested in mixtures with other additives, their mode of action was not deciphered.

The administration of the yeast cell wall in salmonid feeds induced an up-regulation of IL-1β, but no further information about its mode of action could be obtained for this species, as there is only one manuscript exploring this issue. Lipopolysaccharide (LPS) is reported to be highly immunogenic in salmonids, as LPS is a potent activator of complement; however, an overdose of LPS could deplete complement components, leaving the fish more vulnerable to infection, so it is necessary to adjust the doses of LPS and the length of the stimulatory period. Peptidoglycan dietary administration resulted in an up-regulation of many AMPs (antimicrobial peptides) in mucosal tissues (skin, gills, gut) and liver. However, further information is needed concerning the use of the yeast cell wall, LPS, and peptidoglycan in

salmonid feeds in order to better characterise its mode of action and effects on health, as there are just three scientific articles exploring these prebiotics.

4.4.1.3 Salmonids – plant extracts

There is not a clear trend in the preference of testing different plant extracts in salmonids, as the efforts on different compounds may be considered similar (only one manuscript was found for each of the plant extracts considered in this section).

The inclusion of garlic (*Allium spp.*) in salmonids feeds results in an increase in the number of red and white blood cells as well as an increase in the respiratory burst activity and lysozyme. The inclusion of *Thymus vulgaris* derivatives (thymol) affected the total anaerobe counts, which were lower and increased *Lactobacillus spp*. bacteria. Thymol also increased the humoral immune response by increasing the activity of lysozyme, complement concentrations, and catalase activity; it reduced NO serum levels. Similar results were reported when carvacrol was included in experimental diets for salmonids. The dietary administration of an extract of powdered ginger (*Zingiber officinalis*) roots increased phagocytosis; a respiratory burst activity of blood leucocytes, and increased the level of plasmatic proteins. Similar results were observed with *Urtica dioica, Magnifera indica,* and *Viscum album* extracts. In the case of *U. dioica* and *M. indica* extracts, dietary administration was correlated with a reduction in mortality when trout were challenged with *Aeromonas hydrophila*. It is important to mention that, for all of the above-mentioned plant extracts, further information is needed in terms of the active compounds responsible for the physiological responses observed in fish.

The effects of soybean derivatives in the immune competence of different salmonid fish presented in Table 37 should not be considered positive. Signs point to an inflammatory or hypersensitivity response due to the inclusion of anti-nutritional factors in the soybean and, consequently, soybean derivatives cannot be considered immune stimulants, although their content inflavonoids and phenolic compounds may exert some beneficial effects on the activity of antioxidative stress enzymes.

4.4.1.4 Salmonids – animal by-product

Only lactoferrin has been tested as an immune stimulant in diets for salmonids, and no effects on the non-specific immunity and disease tolerance were found in salmon.

4.4.1.5 Salmonids – other substances

Vitamin and algae extracts are two of the most frequently tested feed additives in salmonid fish species. In this context, most of the available literature is related to vitamins C and E. In particular, the combination of β-glucan and high doses of vitamin C had a stimulatory effect on the non-specific and specific immune parameters, and vitamin C enhanced non-specific immune parameters such as phagocyte activities and lysozyme levels, while β-glucan influenced the specific response to an antigen in terms of antibody response. High dietary levels of vitamin E modulated the phagocytic functions of gut leucocytes in rainbow trout. Nevertheless, the role of these two vitamins as natural antioxidant agents should not be neglected although there is no direct information about their mode of action with regard to this issue.

Algae have been tested as a source of feed additives with immunostimulant properties in salmonids. In this sense, a commercial product named Ergosan (a mixture of *Laminaria digitata* and *Ascophyllum nodosum*) modified the organisation of the intestine (increasingvillus height, fold height, enterocyte height, number of goblet cells, and augmenting surface area of the gut mucosa). In addition, it increased some hematological parameters (total white blood cells and red blood cells) and lysozyme activity in the skin mucus, protease activity, and alkaline phosphatase activity.

Supplementation of fish feeds with exogenous nucleotides can have a positive influence on gut structure, disease resistance, immune response, stress tolerance, and vaccine efficacy; however, their mode of action regarding the aforementioned processes has not been analysed in depth.

In vitro studies have revealed that tetradecylthioacetic acid (fatty acid) has anti-inflammatory effects in the ASK cell line (increased expression of anti-inflammatory IL-10) and also attenuates the capacity for production of pro-inflammatory PGE2 while increasing the

expression of Arg1. These in vitro results need to be validated using in vivo nutritional studies, and TTA effects on other compartments of the immune system ought to be tested.

Different minerals have been tested as immune stimulants in fish diets, but as these additives were included in diets as a mixture with other additives their mode of action could not be determined.

4.4.2. Freshwater species

4.4.2.1 Freshwater species – probiotics

Lactobacillus spp. and *Bacillus spp*. were the probiotic bacteria mostly tested in freshwater fish species, representing 59% of the scientific articles published in this area. Similarly to the reviewed literature on salmonids, the mode of action of probiotics in freshwater fish species depends on the study and set of parameters analysed by authors. For instance, *Bacillus spp*. has been reported to modulate intestinal microflora, enhance the activity of digestive enzymes, induce the up-regulation of different cytokines from the intestine (IL-1β, TGF-β and TNF-α), increase the humoral immune response (IgM levels, lysozyme, phagocytic, bactericidal, complement and respiratory burst activities) and the activity of several antioxidative stress enzymes (CAT, SOD, GPX). However, different results were observed across different species of the genera *Bacillus* in front of different pathogenic bacteria, which does not allow for generalising with regard to the mode of action of *Bacillus spp*.

The mode of action of *Lactobacillus spp*. was similar to that described in *Bacillus spp*. with regard to the modulation of the gut microbiota, reducing oxidative stress status, and the enhancement of the humoral immune response (IgM levels, lysozyme, phagocytic, complement and respiratory burst activities), as well as an increase in the immunity of the intestinal mucosa (number of lymphocytes and acidophylic granulocytes in the intestinal epithelium), the number of circulating thrombocytes, leucocytes, and lymphocytes, and the expression of IL-10 and TNF-α. Similar effects were reported with the dietary administration of *Pediococcus acidolactici*.

Contradictory data exists with regards to the use of *Pseudomonas spp*. as a probiotic. For instance, *Pseudomonas spp*. was not recommended in tilapia, yet it improves humoral

immunity (increased lysozyme, alternative complement, respiratory burst activities) and levels of SOD in rohu (*Labeo rohita*).

The administration of inactivated cell suspensions of *Aeromonas spp*. enhanced the cellular and humoral immune responses, although there are contradictory results reporting that cellular rather than humoral immunity is the factor that explains the beneficial effects of these probiotic bacteria. However, the mode of action of such enhancement was not described in the two studies exploring these probiotic bacteria. Similar results regarding the enhancement of the cellular, rather than humoral, immunity have been reported in freshwater fish fed diets incorporating *Carnobacterium spp*.

As described in salmonids, the modulation of gut microbiota has been described in freshwater fish species fed with diets supplemented with *Bacillus spp*., *Lactobacillus spp*., *Lactococcus spp*., Pediococcus *spp*., and *Enterococcus spp*., although this does not mean that the other probiotics tested exert the same effect on freshwater species as there is a gap of information on this parameter in those studies. The use of the yeast *Saccharomyces spp*. modified gut microbiota by increasing the number of bacteria and their diversity as well as increasing the complement component, peroxidase levels, and head kidney phagocytic index.

The dietary administration of *Vibrio spp*. and *Flavobacterium spp*. enhanced the cellular and humoral immune responses, whereas the mode of action of such enhancement was not described in the studies analysing this probiotic bacteria. Regarding the evaluation of other probiotic bacteria, the dietary administration of *Weixella spp*. resulted in an increase of hematological and immunological (total Ig levels) parameters, although very few variables were assayed and therefore few conclusions can be drawn from the study. Similarly, *Rhodopseudomonas spp*. increased humoral immune response (respiratory burst activity) and antioxidative stress parameters (MPO levels, CAT and SOD activities).

In some of the literature analysed, some probiotics (e.g. *Micrococcus spp., Aspergillus spp*.) were tested within a mixture of different probiotic bacteria and consequently it was not possible to decipher their specific modes of action.

4.4.2.2 Freshwater species – prebiotics

The revision of the available literature indicated that prebiotics have been more frequently investigated in freshwater species than in salmonids, even though β-glucan, mannan oligosaccharides (MOS), and fructooligosaccharides (FOS) are the most frequently evaluated prebiotics compounds in this group of fish (56% of the studies explore the above-mentioned additives).

β-glucan has been reported to affect the composition of the intestinal microbial communities in carps and, as a consequence, affect the morphology of the intestine (length of microvilli) and the levels of intraepithelial leucocytes in the anterior intestine, which may indicate a localised immune response. It also stimulated CRP and complement responses to PAMPs immunological challenges. Regarding the effects of β-glucan on molecular markers, this feed additive down-regulated the expression of inflammatory-related genes (IL1β, IL10, TNF-α1, TNF-α2, CXCa and CXCb) in the spleen, head kidney, and gut. Contrary to the results reported in salmonids dietary supplementation of MOS has no effects on haematological parameters, with the exception of lymphocyte and eosinophil levels, which were reduced and elevated respectively. However, the mode of action of this prebiotic is not clear considering the consulted literature on freshwater fish species.

The dietary administration of FOS resulted in an increase of microvilli height in enterocytes, as well as modulating the humoral (high levels of Ig, serum lysozyme, and alternative complement activities) and cellular (increased white blood cell and lymphocyte levels) immune responses. Similarly, yeast wall cells improved gut morphology and modulated the humoral and cellular immune response. The inclusion of galactooligosaccharides (GOS) has been reported to modulate the intestinal microbiota by increasing lactic acid bacteria and increasing stress tolerance, but the effects of GOS on immune parameters have not been evaluated. Similar results have been obtained by the oral administration of arabinoxylan oligosaccharides (AXOS), which have been reported to modulate the intestinal microflora that may enhance the production of short-chain fatty acids that would benefit the host. AXOS has also been reported to enhance immune function, although this additive's mode of action has not been described. Similarly, the administration of chitooligosaccharides (COS) has been reported to enhance fish immunity (cellular and humoral response) by increasing

leucocyte counts as well as respiratory burst activity, lysozyme activity, SOD, and phagocytic activity. Contrary to the other oligosaccharide compounds tested, xylooligosaccharides (XOS) increased mucus antibacterial activity and total protein levels; it also modulated gut microflora by increasing the number of lactic acid bacteria and heterotrophic bacteria, but no effects on intestinal morphology were observed.

When used as a feed additive, Levan increased haemoglobin content, lysozyme activity, and respiratory burst activity. The inclusion of inactivated yeast-bacteria has been reported to modulate gut microbiota, but no information is available on its effects on the immune function of fish. Further information is required on the aforementioned feed additives, as the literature on their effects on the immune system and overall condition of fish is currently limited. Other prebiotic compounds, such as inulin and lipopolysaccharide (LPS), have been tested in fish diets; this has been done as a mixture with other additives, so their individual modes of action could not be determined.

4.4.2.3 Freshwater species – plant extract

The number of studies evaluating different plant extracts in freshwater fish species are almost double the number of studies on salmonids. There is a scarcity of studies dealing with a similar compound; all the information for each of the plant extracts tested is presented in just one manuscript, with the exception of *Achyranthes spp*. derivatives, which has three. Thus the inclusion of *Achyranthes* spp. derivatives the diets of different Indian carp resulted in a higher serum of protein and globulin levels as well as myeloperoxidase activities. The inclusion of *Thymus vulgaris* derivatives (thymol) affected the blood phagocytic activity but not the level of respiratory burst activity or the lysozyme activity. Unlike the information available about salmonids, there is no data on the effects of thymol on gut microbiota, so a similar effect in freshwater fish species cannot be stated with certainty.

The inclusion of propolis in the diets of freshwater fish species enhanced the humoral immune response by increasing the serum and mucus lysozyme activity. Propolis was tested, and evidence indicates that its ethanolic-extract was more effective than crude propolis in protecting fish against infection. In this case, propolis enhanced macrophage-functions and lymphocyte proliferation, as well as the serum bactericidal and lysozyme activities; this meant increased resistance to several pathogens. Similarly, the use of mango kernel

(*Magnifera indica*) and *Astragalus* polysaccharides also enhanced humoral immune response by increasing the production of the superoxide anion, lysozyme, serum bactericidal activity, and serum protein and albumin levels.

Alnus firma, Trigonella spp. and *Rosmarinus officinalis* derivatives, as well as raw fibre, have been reported to increase the number of white and red blood cells, neutrophils, and monocytes, as well as the phagocytic activity of neutrophils and an increase in serum lysozyme, haematological, and immunological changes that were coupled with a decrease in mortality when fish were challenged with *Streptococcus iniae*. Other plant derivatives, such as those obtained from *Withania sommifera,* have been shown to enhance the humoral immune response (respiratory burst activity, lysozyme, Ig levels) that increased the survival of fish challenged by *Aeromonas hydrophila*.

Other plant-derived compounds like those originating from *Oscimum sanctum*, *Azadirachta indica* and *Curcuma longa* have been tested in fish diets as a mixture with other additives, so their mode of action could not determined.

4.4.2.4 Freshwater species – animal by-products

As with salmonids, there is little edata available on the use of animal by-products as feed additives for enhancing the immune condition of freshwater fish, which may be due to the tight regulation involved in using these substances. Unlike in the study conducted in salmonids, lactoferrin dietary administration in catfish resulted in an enhancement of the humoral immune response (increased serum lysozyme level, oxidative radical production) that meant increased protection from *Aeromonas hydrophila* challenge. In addition, the administration of chitooligosaccharides / chitosan-oligosaccharides obtained from shrimp significantly reduced the inflammatory response and stress response in tilapia intestine, as evidenced by lower levels of mRNAs encoding for TNF-α and HSP70 and a higher level for TGF-β.

4.4.2.5 Freshwater species – other substances

As withs almonids, vitamins and *algae* derivatives were the most commonly tested feed additives with immune stimulant properties tested in freshwater fish species. However, a

larger variety of vitamins has been tested in freshwater fish species than in salmonids. Vitamin C enhanced both the non-specific and specific immune responses in Indian carp by increasing serum parameters, respiratory burst activity, lysozyme activity, bactericidal activity, and antibody titers. The influence of vitamin C on immune response may also be related to its antioxidant activity as a free radical scavenger, protecting cells from auto-oxidation and maintaining their integrity, potentially leading to optimal functioning of the immune system. Supplementation of vitamin E in the diet seems to protect the complement system from stress-related reduction of activity, whereas choline enhanced serum activities of lysozyme and ACP, haemagglutination titer, C3 and C4 levels, and leucocytes phagocytic activity of carp after a challenge suggest an improvement in innate immune response. IgM and anti-AH antibody titer in serum were enhanced by choline, and similar results have been reported with vitamins B6 and B5, for which enhancement of the humoral immune response was reported.

Regarding the use of algae derivatives, the effects of Ergosan in freshwater fish species was similar to that found in salmonids, especially in terms of enhancing the activity of lysozyme, lymphocyte, and neutrophil counts. The incorporation of *Chlorella spp*. has been reported to increase the total protein albumin and globulin content in the serum, as well as the activities of SOD and lysozyme, thus affecting the humoral immune response. Feeds incorporating *Dunaliella salina* as a food additive resulted in an increase in red and white blood cells, but the mode of action of this microalga or its active compounds is unknown.

The administration of apidaecin-type peptides induce positive modulation in the immune response, likely through regulating the intestinal microflora, inhibiting the growth of gram-negative potential pathogens and thus stimulating the growth of intrinsic probiotics. In addition, apidaecin increased serum lysozyme activity, thus having a direct effect on the humoral immune response.

Amino acids have frequently been evaluated for their potential immunostimulant properties, and among these there is available information for four types. In this sense, methionine promoted humoral immune response by increasing plasma lysozyme activities, lectin potency, and IgM, C3 and C4 contents. Similarly, methionine hydroxyl analogue improved disease resistance by increasing the growth of immune organs and humoral immune response whilereducing lipid or protein oxidation in the head kidney and spleen in order to

protect the structure and function of immune organs. Dietary tryptophan enhanced innate intestinal immunity, barrier function, and antioxidant status while attenuating intestinal inflammatory response. This amino acid exerted an anti-inflammatory effect by up-regulating the expression of IL-10 and TGF-β1 and down-regulating TNF-α and IL-8, which may be related to the down-regulation of TOR in the intestines of fish. Trp also up-regulated the gene expression of occludin, ZO-1, claudin-b, -c, and -3, and down-regulated the gene expression of claudin-12 and -15, improving the intestinal barrier function of fish. Dietary Trp enhanced the SOD1 and GPx activities and mRNA levels in the intestines of fish, which may be associated with the Keap1-Nrf2 signalling pathway. Dietary arginine and glutamine supplementation tended to improve neutrophil oxidative radical production and intracellular superoxide anion production in head kidney macrophages, but it also increased the humoral immune response (lysozyme activity and extracellular superoxide anion production) and promoted the growth of the intestinal epithelium (fold height in the mid- and distal intestine and microvillus height in the pyloric caeca).

The role of minerals (chromium) as immunostimulants in different freshwater species has been analysed, but no clear conclusions can currently be made based on these studies as it is sometimes unclear whether the results correspond to an effect derived from a potential toxic effect of mineral accumulation or is due to its properties as an immune stimulant.

Silage oils have been described as improving cellular non-specific immunity and simultaneously decreasing total mortalities in tilapia; this has been attributed to these oils' antimicrobial effects of improving the phagocytic activity of leukocytes.

Other compounds, such as extracellular extracts of *Aeromonas veronii* and *Flavobacterium sasangense*, poly-b hydroxybutyrate-hydroxyvalerate (PHB-HV) from *Bacillus thuringiensis* or N-acyl Homoserin Lactonase from *Bacillus spp*. strain B546, were found to have significant immunostimulatory effects on both specific and non-specific immunity (antibody response, lysozyme activity, number of neutrophils), which may be due to the presence of PAMP in these extracts.

4.4.3. Marine fish and Shellfish species

4.4.3.1 Marine fish and Shellfish – probiotics

As with salmonids and freshwater fish species, *Bacillus spp.* and *Lactobacillus spp*. were the probiotic bacteria mostly frequently tested in shellfish and marine fish species; these represent 53% of the scientific articles published in this area.

The addition of *Bacillus spp*. to the water modulated the bacterial growth in shrimp gut by competitive exclusion and by boosting immune responses (increase of phenoloxidase, lysozyme, respiratory burst, bactericidal and nitric oxide synthesise activities), hemocyte count, and plasma protein levels. In addition, *Bacillus spp.* increased the activity of digestive enzymes, although the mode of action was not assessed. In other crustaceans, such as marron and lobster, *Bacillus spp.* administration has been reported to modulate the microflora, improving the overall condition of animals. The aforementioned effects were also observed in sea cucumbers (equinoderm). In marine fish, *Bacillus spp*. enhanced both cellular and humoral immune responses (phagocytic, lysozyme, peroxidase, complement, SOD and peroxidase activities), as well as the up-regulation in the expression of IL-8, CASP-1, COX-2, and transferrin. These physiological changes were coupled with a modification of the microbiota in the intestine. Similarly to *Bacillus spp*., *Psychrobacter spp*. and *Vagococcus spp*. enhanced the specific activity of digestive enzymes, improving feed utilisation humoral immune response (SOD, phagocytic and complement activities), and the up-regulation in the gene expression of TLR2, TLR5, MyD88, and several cytokines (IL-1β, IL-8 and TGF-β1).

In marine fish, *Lactobacillus spp*. also had an effect on gut microflora (increase in lactic acid bacteria), resulting in an increment of T-cells an increment of Ig+ cells and acidophilic granulocytes, and an increment of the digestive capacities. *Lactobacillus spp*. has been reported to enhance CAT and SOD activities, as well as phagocytiic, respiratory burst, complement, lysozyme, and cytotoxic activities. Some articles assessing different molecular markers have reported that this probiotic up-regulates the expression of pro-inflammatory (IL+1β, IL-6, IL17a/f-3, TNF-α, TNF-n) cell-mediated immunity- inducing (IL-12p35, IL-12p40 and IL-18) antiviral / intra-cellular pathogen killing (I-IFN-1 and IFN-γ), anti-inflammatory (IL-10), and peripheral T cell expansion and survival controlling (IL-2, IL-7, IL-15, IL-21 and TGF-β1) cytokines. With regard to shrimp, *Lactobacillus spp*. also enhance

several immune parameters such as total hemocyte count, phenol oxidase (PO) activity, respiratory burst activity, SOD activity, proPO, and PE mRNA transcription, although this probiotic disappeared from the gut of shrimp when its dietary administration was interrupted.

Pediococcus spp., Arthrobacter spp., Aeromonas spp. and *Enterococcus spp.* enhanced humoral immune function (IgM levels and phagocytic, lysozyme and respiratory burst activities) in shrimp and grouper, with an inhibitory potential toward pathogenic *Vibrio spp*. Similar results have been obtained with *Lactococcus spp*., showing a strong antibacterial activity against pathogenic bacteria such as *Streptococcus iniae*, *S. parauberis*, and *Enterococcus viikiensis*. Other probiotic bacterial species, such as *Shewanella spp*., have been described as sources of natural antioxidative compounds as well as enhancers of cellular and humoral immune responses. Although some promising results were obtained under in vitro conditions, these must be validated in vivo and under fish rearing conditions. Regarding *Clostridium spp., Streptococcus spp. and Pseudomonas spp*., their dietary administration enhanced immune response (phagocytic activity of head kidney leucocytes, total Ig level in serum and gut mucus, serum phenoloxidase activity, and acid phosphatase activity) as well as the level of lysozyme, hepcidin, transferrin and metallothienin, respectively. However, depending on the species (fish vs. shrimp) different probiotic effects were reported; no probiotic effects of *Pseudomonas spp.* in shrimp have been reported.

It is important to note that there are just a few articles exploring the probiotic effects of *Aeromonas spp., Streptococcus spp., Alteromonadaceae spp., Halomonas spp., Phaebacter spp., Hanseniaspora spp.,* and *Zooshikell spp*. (generally just one article per genera of bacteria). Consequently, even though most of these studies reported an increase in the humoral immune response in shrimp or marine fish, their results could not be contrasted with those of other studies, and thus their mode of action should be understood with caution.

The use of the yeast *Saccharomyces spp*. induced the up-regulation of innate cellular and humoral immune responses (SOD, phagocytic, and respiratory burst activities in head kidney leucocytes, as well as levels of serum lysozyme, complement activity, and citotoxic activity). In shrimp, *Saccharomyces spp*. has been reported to increase serum lysozyme, as well as improving the quality of the sediment in shrimp rearing ponds (improvement of rearing

conditions). Another yeast tested as a probiotic is *Debaromyces spp.*, which has been reported to modulate the intestinal microbial community and increase the non-specific humoral immune response (alkaline phosphatase activity) in shrimp, as well as improving the cellular immune function in fish. In addition, *Debaromyces spp*. up-regulated the expression of several genes (IgM, MHC-Ia, MHC-IIa, C3, IL-1β, TLR, TNF-α, CSF-1R, NCCRP-1, Hep, TCR-β and CD8 genes) in the skin, intestine, and head kidney.

Other tested probiotics, such as *Paffia spp.* or *Aspergillus spp.,* did not have the desired probiotic effect.

4.4.3.2 Marine fish and Shellfish – prebiotics

The same trend observed in the experimental administration of prebiotics in marine fish and shellfish has been detected with regard to these compounds tested in salmonids and freshwater fish species; these additives include β-glucan, mannan oligosaccharides (MOS), and fructooligosaccharides (FOS) as the most frequently studied (73% of the scientific articles explore these three additives). It is interesting to note that prebiotics were more frequently studied in shellfish and marine fish species than they were in freshwater fish and salmonids.

It seems that β-glucan modulated immune response by increasing phagocytic activity and IL-1β and decreasing IgM in fish. In marron (crustacean species), β-glucan increased the total haemocyte count and granular cells, whereas it increased plasma protein, haemocyte count, phenol oxidase activity, superoxide anion production, alkaline phosphatase, and acid phosphatase activity in shrimp. As it did in fish, β-glucan indirectly affected the response of the host to pathogen by reducing the level of the anti-inflammatory cytokine IL-10 and increasing IL-1β.

As with salmonids and freshwater fish species, the administration of MOS in feed directly affected the gross morphology of the intestine by promoting its development and increasing the intestinal barrier (enlarged intestine folds height and width due to the enlargement of the lamina propria and the promotion of the development of gut-associated lymphoid tissue, GALT). In addition, MOS also enhanced the cellular and humoral immune response, increasing the expression of IL-1β and IL-8. No data is available on the effects of MOS on

the microbial community in marine fish or shellfish species. In sea cucumbers (echinoderm species), the administration of FOS resulted in total coelomocyte counts and phenoloxidase activity and could enhance phagocytosis as well as potentially modulating gut microflora. In shrimps, FOS altered the gut microflora and enhanced the humoral immune response (respiratory burst activity of hemocytes). In contrast, FOS administration in fish was detrimental in terms of the organisation of the digestive tract, but it increased the levels of haemoglobin, leucocytes and lymphocytes, although the correlation of these values to a higher immune competence of fish has not been clearly established at this time. Other tested oligosaccharide compounds in marine fish and shellfish are galactooligosaccharides (GOS) and transgalactooligosaccharides (TOS), which have been reported to increase the humoral immune response (lysozyme), affect the composition of the microbiota in the case of GOS, change the morphology of the intestine (increase the length of the intestinal folds, enterocyte's size, and microvilli size), and modulate the activity of digestive enzymes in the case of TOS.

The administration of yeast cell walls in the diets of marine fish and shellfish enhanced immune response, as well as the organisation of the intestine (enlarged intestine folds height and width, increase in microvilli height, number of goblet cells in the intestine, and the production of mucus, thus enhancing this protective barrier in the gut) and the activity of the digestive enzymes. Although the effects of this feed additive on gut morphology were well characterised, their mode of action on the immune response was not addressed in detail. The administration of chitin enhanced the humoral immune response by increasing the activity of the haemolytic complement and respiratory and cytotoxic activity in leucocytes in fish.

Contradictory findings have been found regarding the use of inulin when comparing data obtained from fish and shrimp. In particular, the combination of *Bacillus subtilis* and inulin exerts a negative effect on gilthead sea bream gut homeostasis, and to date immunostimulant effect of inulin on the innate immune system has not been found in this marine fish species. In contrast, inulin has been reported to reduce the prevalence of white spot syndrome (WSSV) in shrimp, although its mode of action has not yet been described.

4.4.3.3 Marine fish and Shellfish – plant extracts

Plant-derived extracts have been more frequently studied in marine fish and shellfish species than into salmonids and freshwater fish species, not only in term of the number of different compounds but also in terms of the number of studies. However, similarly to the other group of fish species, there is generally only one scientific study per plant-derived compound.

Allium spp. derivatives in diets for marine fish enhanced the humoral immune response, as was reported in salmonids species. This immune enhancement was correlated with a lower mortality in fish exposed to *Edwarsiella tarda*. Similar results were reported for *Zingiber officinalis*, which were reported to increase haemoglobin content, respiratory burst activity of neutrophils and serum lysozyme, and the number of white blood cells. These results are in accordance with those reported by *Kalopanax pictus,* in which the enhancement of the humoral immune response (higher levels of respiratory burst activity, lysozyme activity, bactericidal activity, total protein, and myeloperoxidase) was coupled with increased resistance to the pathogenic bacteria *Vibrio alginolyticu*s as well as increased resistance tothe parasite (*P. dicentrarchi*). Similar results were reported with the dietary administration of *Broussonetia kazinoki*; there was an enhancement in the humoral immune response (increase of phagocytic, complement, lysozyme, and respiratory activities) accompanied by increased resistance to an infection of *Streptococcus parauberis*. In this sense, the inclusion of *Lactuca indica* has been reported to increase humoral immune function, but this finding was not correlated with an increased tolerance to a pathogen, as no bacterial challenge was conducted in the study analysed.

The inclusion of *Citrus* by-products, in combination with probiotics, has been reported to increase myeloperoxidase and lysozyme activities; these results were translated into increased disease resistance to *E. tarda*. However, further information is needed about the mode of action of this product and its combined effects with probiotics.

The use of *Mentha piperita* in the diets of marine fish and shellfish species resulted in increased phagocytic activity, which significantly improved the non-specific immunity of fish. In addition, the main active component of *M. piperita* leaves is the essential oil that contains a number of bioactive compounds (such as phenolic, tannins, and flavonoids) known to exhibit biological activities, especially antimicrobial activities. This is also an effective

antioxidant and antiperoxidant compound, which triggers the production of a superoxide anion. These properties have been correlated with the increased survival of fish exposed to pathogenic bacteria. Other plant-derived products, such as those obtained from *Phoenix dactylifera,* have been reported to be a good source of natural antioxidants, as these regulate the transcription levels of genes coding for SOD, CAT, and GR, although no information is available on their mode of action on conventional cellular and humoral immune parameters.

The effects of soybean derivatives and linseed oil on the immune competence of different marine fish species are presented in Table 39; as with the effects considered for salmonids, this should not be considered a feed additive for enhancing the immune system and fish condition but rather a collateral effect of including soybean and linseed oil in diets as alternative fish meal and fish oil sources. Consequently, the mode of action of this raw material is not further discussed in this section.

Several plant-derived compounds from *Uncaria tomentosa* and *Echinacea spp.* exist which contain molecules with immunostimulant properties, such as caffeic acid derivatives, polysaccharide fractions, alkamides, quinovic acid glycosides, and pentacyclic oxindole alkaloids. However, their mode of action on the animals' condition and immune status has not been addressed in the publication exploring these compounds.

Other plant-derived compounds, such as those originating from *Cucurbita moshata*, *Lonicera japonica*, *Panax ginseng*, *Carthamus tinctorius*, *Plantago asiatica, Glycyrrhizaspp.*, *Echinacea spp.*, *Aconitum koreanum,* and *Oscimum sanctum* have been tested in fish diets in a mixture with other additives, so their individual modes of action could not be determined.

4.4.3.4 Marine fish and Shellfish – animal by-products

Lactoferrin administration has been described to have a direct but weak effect on leucocytes in vitro studies, whereas for *in vivo* studies it activates the cellular innate immune response by enhancing respiratory burst and leucocyte-mediated cytotoxic functions. These results are somehow different from those reported in salmonids and freshwater fish species, which may be due to different experimental approaches and analysed variables. This means that it is not currently possible to draw general conclusions on the impact of lactoferrin on fish.

Fish protein hydrolysates have been reported to improve the production of IgM and lysozyme activity as well as to modulate the gut microbiota; these parameters were associated with an improvement in the resistance of fish to *Vibrio anguillarum* challenge.

Antibodies (IgY from egg powder) have been tested in abalone (mollusc species) and have shown to increase the respiratory burst activity and prevent infection from *Vibrio alginolyticus*. No information about their use in fish and crustaceans is currently available.

4.4.3.5 Marine fish and Shellfish – other substances

Studies exploring *algae* were the most common in this section. Regarding marine fish and shellfish species, the immune stimulant effects reported for vitamin C and E in this group of animals is similar to that reported previously in salmonids and freshwater fish species.

As it did in salmonids and fresh water fish species, the use of Ergosan as a feed additive had an effect on marine fish and shellfish species. In addition, the use of different microalgae, such as *N. gaditana*, *T. chuii*, and *P. tricornutum,* stimulated certain activities of the gilthead sea bream immune system and slightly affected immune-related gene expression, although these effects differed among microalgae species. In particular, *Phaeodactylum tricornutum* has a higher enhancing effect on immune parameters, which might be attributed to the presence of a cell-wall-sulphated glucomannan and a β-1,3-glucan. Microalgae increased the expression of CSF-1R and MHC-IIa genes in the gut. Dietary applications of *B. subtilis*, *T. chuii*, and *P. tricornutum*, on their own or in combination, may exhibit up-regulating effects on gilthead sea bream immune parameters (complement activity, IgM level, respiratory burst, and phagocytic activity). Other microalgae, such as *Navicula* spp. and *L. sakei,* have been reported to increase the humoral immune response by elevating the levels of myeloperoxidase, lysozyme, total antiproteases activities, and IgM. Regarding this type of feed additives in shrimp, data indicates that shrimp fed the *Gracilaria tenuistipitata* extract-containing diets showed increased immunity by increasing circulating haemocytes, PO activity, RBs, SOD activity, GPX activity, and lysozyme activity together with increases in mitotic cells; this was correlated with an increased resistance to *Vibrio alginolyticus* and WSSV infections. In addition, shrimp fed with *Sargassum fusiforme* polysaccharide extract (SFPSE) displayed significantly lower cumulative mortalities and enhanced immune activity (THC, PO, and LSZ activities).

Regarding minerals, the oral administration of Barodon, a commercial product (Anionic Alkali Mineral Complex), was reported to affect the membrane-associated lymphoid tissues, as well as the innate immune response, through the increase in lysozyme, MPO and SOD activities.

The effects of different types of feed additives, such as amino acids (arginine, glutamine), peptides, nucleotides, and fatty acids reported in freshwater species were similar to those observed in marine fish species. In addition, the dietary administration of acidic calcium sulphate increased the phagocytic activity in hemocytes and elevated total hemocyte counts in shrimp, resulting in an increase in stress tolerance.

As isomaltooligosaccharide (IMO) was tested in fish diets containing a mixture of other additives, its mode of action could not be determined.

4.5. Rabbits

As meat producing animals, rabbits are considered a minor species; this fact could explain the limited studies conducted on immune stimulator substances/agents that are likely to being used as feed additives in rabbit diets. Additionally, in the reviewed papers, the diversity of parameters used for the evaluation of the additive effects is very high, increasing the difficulty in inferring the most useful analyte / parameter to be systematically included in controlled or field type studies.

Furthermore, the reduced number of papers on rabbits (13) also means a lack of repetition which could have been helpful for the evaluation of the reproducibility (and consequently the strength) of the results under different productive conditions. As we can infer from the analysed papers, there is limited information on the effects of probiotics and prebiotics on rabbit health, and solid conclusions cannot be made at this point in time.

4.6. Equine

The total number of articles found in the systematic review using horses as a target animal is limited. Only four articles studied the effects of probiotics included in the diet of horses, and these were conducted primarily to assess the capacity of colonisation bacteria that is 'beneficial' to the intestinal tract. *Lactobacillus spp.* is able to colonise the intestine of foals. The worst results were founded in adult horses; the presence of this organism in the faeces of adult horses may only represent passive movement through the intestinal tract and may not be due to colonisation. Supplementing equine diets with direct-fed LAB (blend of different probiotics) had a limited effect on nutrient digestibility or reducing the risk of acidosis associated with feeding high-starch concentrates to horses. The inclusion of *Saccharomyces cerevisiae* in the diet did not yield statistical significance in the improvement of horses after enterocolitis.

The searches only yielded two articles about prebiotics in horses. These studies assessed FOS, GOS, and AOS effects on innate immunity. FOS supplementation could be effective in reducing disruptions of the microbial populations in the equine hindgut under stressful situations, such as acute starch overload. FOS could lead to a beneficial increase in VFA production, such as butyrate.

Oligosaccharides from different sources have distinct and direct immunomodulatory properties. Using a blend of prebiotics (GOS and GOS / FOS fractions) that were dependent on dose enhanced the pro-inflammatory response (TNF-α) in LPS-challenged equine PBMCs. In contrast, after incubation with 2% GOS/FOS/AOS, the LPS induced inflammatory response was dose-dependently reduced. Increasing the AOS concentration in the incubations meant that the stimulatory effects of both GOS and GOS / FOS were eventually overruled by the suppressive effects of AOS. Such an activation or suppression of the immune system could be beneficial in vivo, depending on the clinical context.

Only one plant extract was evaluated as a component of a mixture with other probiotics in horse feed. This blend enhanced faecal sand clearance in normal horses, but the specific activity of psyllium could not be determined.

Two articles studied the effects of minerals on the guts of horses. Baradon (a blend of minerals) is a potential immunostimulant and alternative to antimicrobial feed additives for improving equine immune responses. Its use seems to result in the improved capability of horses to endure an attack of infectious respiratory diseases. Baradon induces higher proportions of cells expressing major histocompatibility complex class II and CD2, CD4, CD4-CD25, CD8, and CD8-CD25 T-lymphocytes, dendritic cells, and surface immunoglobulin M and B-lymphocytes in peripheral blood, as well as enhanced cell proliferative responses and increased phagocytic activity against bacteria. Conversely, Selenium status (either high or low) did not affect the ability of the horses to display an immune response to a novel antigen, with the exception of IL-10 expression.

4.7. Pets

Probiotics are used in clinical practice as tools to manage animals with diarrhoea or other gastrointestinal diseases; in some cases, they are also used in healthy animals, with the hope that the result will be a healthier gastrointestinal tract and the prevention of disease. Evidence of these actually helping in such cases is relatively scarce, although these products can be found on the market both alone and in combination (synbiotics) as well as in some pet foods.

As seen in the results, the effects of the additive on immunity varied depending on the probiotic used. In cats, *Enterococus* was the most effective (stable microbiome, lower morbility associated with feline herpes virus-1 in some cats), while both *Lactobacillus* and *Enterococcus* yielded positive results in dogs. The mode of action of these probiotics is not always described in the literature, but *Lactobacillus* exerted beneficial effects in dogs, in terms of cytokine expression, primarily through regulatory T-cells (up-regulation predominantly of IL-10 and to a lesser extent of TGF-1, leading to an overall decrease in the pro-inflammatory / regulatory cytokines ratio). *L. casei*, expressing the cGM-CSF protein, stimulates the production of monocytes and CCV-specific IgG in CCV vaccinated dogs. These also reduce ammonia formation, increase lactic acid, and reduce other bacteria (likely due to the acidification of the GI tract by lactic acid).

As for *Enterococcus faecium* in cats, there was no detectable enhancement of FHV-1 serum IgG antibody responses or lymphocyte responses to concanavalin A or FHV-1 antigens in SF68-supplemented cats. In dogs, however, they increased both Ig synthesis and TNF.

The end-points measured for probiotics have several limitations. Microbiota was the most frequently studied end-point (14 articles of a total of 21); however, most canine research (particularly research sponsored by pet food companies) is non-invasive and thus faecal microbiota is the most common analysis. It is not possible to affirm that faecal microbiota is reflective of what happens in the colon or in earlier parts of the gastrointestinal tract. Other responses, such as complete blood cell count, are very difficult to interpret as they primarily fall within the reference ranges, despite any changes. The increase in Ig, especially before vaccination, is the optimal end-point regarding the effect of probiotics on immunity. However, the number of studies is small (three articles of a total of 21). Performance in pets is also difficult to measure, as pets are not used for the production of food. Health status is also rarely measured, except for diarrhoea in some cases (not defined in 16 articles out of the total of 21).

Prebiotics are commonly used in pets, either separately or as part of combinations (synbiotics) or within the diet. The majority of diets include fibre sources, and some of these include prebiotic fibre sources such as chicory root (source of inulin which is, in turn, a source of FOS), sugar beet pulp, and *Platago spp*. The inclusion of fibre can result in better faecal quality and can promote satiation in pets, up to a certain point.

In terms of immunity, the most frequently studied prebiotic is FOS, followed by MOS. Inulin (a source of FOS) and the yeast cell wall (a source of MOS) has also been studied. In cats, there is no clear result of FOS inclusion, except for an increase in *Bifidobacterium*. In dogs, this prebiotic (and inulin) does affect bacterial composition (mostly in faeces) and can alter the products of bacterial metabolism, reducing putrefactive compounds, and increasing propionic acid. This also increases Ig concentration in milk and colostrum, and one study indicated partial protection against infection. The mode of action is thought to be via the selective growth of beneficial bacteria, which would then act like probiotics (competing with pathogens, producing lactic acid, and affecting cytokine synthesis, etc.). As for MOS (and the yeast cell wall), in dogs these tend to increase *Lactobacillus* and decrease anaerobes, likely due to promoting *Lactobacillus* growth, which then competitively inhibits the growth of other

pathogenic bacteria. It also helps to manage diarrhoea due to EPEC, and this effect is likely due to the fact that MOS bind to type-1 mannose site in *E. coli*, preventing the bacteria from binding the enterocytes.

Prebiotics, like other fibres, can result in decreased digestibility; this is a negative side effect, the degree to which will depend on the dose and may not be significant.

End-points are quite restrictive in dogs and cats. Most microbiota are measured in faeces (with the aforementioned limitations; 13 articles out of a total of 17) and, as with probiotics, changes in complete blood cell count and Ig are difficult to interpret (six articles out of a total of 17).

At this time, there are no clear results for Lactoferrin and plant extracts with regard to feline and canine immunity.

5. General remarks

1. Articles included in this systematic search typically used the term "immunomodulation" rather than "immunostimulation". The term "modulation" does not imply an induction of an immunological parameter. The beneficial effects of dietary feed additives on animal health may be caused by down- or up-regulation of a specific immune parameter.

2. Some substances / agents included in this study have already been considered as feed additives by the European Commission. Currently, some DFM, vitamins, amino acids, minerals, and enzymes are regulated by Regulation (EC) No 1831/2003, as zootechnical or nutritional additives.

3. In this revision, few interactions between dietary composition and substances / agents were noted. The main interactions studied were those between two or more immunomodulatory substances / agents added to the diet.

4. A reduced number of studies evaluated the safety of the additives, likely because the main purpose of this systematic review was to identify efficacy rather than check the safety of substances / agents with immunomodulatory effects making them likely to be used as feed additives.

5. A large number of reports explore mixtures with more than one immunomodulatory substance / agent. Therefore a specific mode of action identified in these studies cannot be attributed to any individual compound.

6. The mode of action of some plant extracts is poorly investigated, either because these plants were studied primarily within mixtures or because few studies are available.

7. Few studies assess the dose-response of substances / agents on immune parameters. Dosages may have a significant influence on the results and the interpretation of data. Tables of mode of action do not include specific responses to dose for each substance / agent due to a wide range of applications (i.e. route of feeding, dosage, time, age of animal).

8. Minerals, amino acids, and certain vitamins are common compounds in diets. These products can only be considered as immunomodulator substances when the dosage is above the minimum requirements established.

9. Taking into account the biological properties of the substances / additives investigated, improvements in the performance of the parallel, with the end-points, are those that might be considered indicatives of animal welfare.

The first remark indicates that the term "modulation" does not imply an induction of an immunological parameter. Klasing (2007) suggested that the term "stimulate" should be used only in its immunological context of causing the immune system to respond. This term is used differently by immunologists and pathologists than it is by nutritionists. For this reason, the International expert advisory panel involved in the project suggests the following definition for immunomodulatory substances / agents:

Immunomodulator:

"Non-nutritional and non-essential nutritional compounds / organisms that improve the protective efficacy of the intestinal innate and / or adaptive immune system under challenge and / or stress conditions excluding nutritional deficiencies."

6. References

The references for the bibliography used in the systematic review included in level 3 of DistillerSR (1144 articles) are available in appendix A

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7. Abbreviations

AA	Amino acid
ABP	*Achyranthes bidentata* polysaccharides
ACH50	Alternative complement activity
ACP	Alternative complement pathway
AcP	Acid phosphatase activity
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
AF	Aflatoxin
AG	Arabinogalactan
AGP	Antibiotics Growth Promoters
Agp	Acid glycoprotein
AGs	Acidophilic granulocytes
AiiAB546	N-acyl Homoserin Lactonase from Bacillus spp. B546
AKP	Alkaline phosphatase
AL	*Artemisia* leaves
AMP	Antimicrobial peptide
AOS	Arabinoxylan oligosaccharide
APC	Antigen Presenting Cells
APE	*Agrimonia procera* extract
APS	*Astragalus* polysaccharide
Arg	Arginine
ASC	Antibody secreting cells
ASK	Atlantic salmon kidney
ATM	*Astragalus membranaceus* root

AvBD9:	Avian β-defensin 9
AX	Arabinoxylan
AXOS	Arabinoxylan oligosaccharide
BA	Butyric acid
BCFA	Branched-chain fatty acid
Bcl	B-cell lymphoma
BCS	Black curcumin seeds
BE	Berberine
BHV-1	Bovine herpesvirus 1
bLF	Bovine Lactoferrin
BT	*Brevibacillus texasporus*
BUN	Blood urea nitrogen
BW	Body Weight
BWG	Body Weight Gain
BWI	Body Weight Increase
Ca	Calcium
CASP	Caspase
CAT	Catalase
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine (C-C motif) ligand
CCV	Canine Coronavirus Vaccine
CD	Crypt depth
CDV	Canine Distemper Virus
CE	Cellooligosaccharide
CFB	Complement factor B

CFIA	Canadian Food Inspection Agency
CFU	Colony forming units
CGF	*Chlorella* growth factor
cFM-CSF	Canine granulocyte macrophage colony stimulating factor
cGRP	Calcitonin gene related peptide
CHLO	Cholesterol
CLA	Conjugated Linoleic Acid
CLS	Coelomocytes lysate supernatant
CMH	Chinese Medicinal Herbs
Con	Concanavalin
COS	Chitooligosaccharide
COX	Cyclooxygenase
CP	Crude protein
CPC	Cooperative Patent Classification
Cr	Chromium
CRP	C-reactive protein
CTLA	Cytotoxic T-Lymphocyte Antigen
CTTAD	Coefficient of total tract apparent digestibility
CSC	Chemokine (C-X-C motif)
CSF	Colony stimulating factor
CXCL	Chemokine (C-X-C motif) ligand
CYP	Cytochrome
Cys	Cysteine
DAO	Diamin oxidase
DBT	Dangguibuxue Tang

DC	Dendritic cell
DCP	Dried *Chlorella* powder
DFA	Difructose anhydride
DFM	Direct Feed Microbial
DGGE	Denaturing Gradient Gel Electrophoresis
DHC	Different haemocyte count
DM	Dry Matter
DNA	Deoxyribonucleic acid
DFI	Daily Feed Intake
DWG	Daily Weight Gain
EAC	Erythrocyte-antibody complement cells
EC	European Commission
ECG	Eosinophilic granulocytes
EFSA	European food Safety Authority
EFO	Epimedium polysaccharide (EP)-propolis flavonoid (PF) oral liquid
EGF	Epidermal growth factor
EMB	Emamectin benzoate
EO	Essential Oil
EOM	Essential oil mixture
EP	Epimedium polysaccharide
EPEC	Enteropathogenic *E. coli*
ERFC	Erythrocyte rosette-forming cells
ERK	Extracellular signal-regulated kinase
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union

FA	Folic acid
FCR	Feed Conversion Ratio
FE	Feed Efficiency
FEEDAP	Scientific Panel on Additives and Products or Substances used in Animal Feed
FG	*Aspergillus* niger-fermented-*Ginkgo biloba* leaves
FHV	Feline Herpes virus
FLC	Fresh liquid *Chlorella*
FO	Fish oil
FOS	Fructooligosaccharide
FPH	Fish protein hydrolysates
FSANZ	Food Standards Australia New Zealand
FSBM	Fermented soybean meal
FSCJ	Food Safety Commission Japan
FSE	*Forsythia suspense*
FUC	Fucoidan
GAC	Gut active carbohydrates
GALT	Gut-associated lymphoid tissue
GC	Goblet Cell
GGMO-AX	Galactoglucomannan oligosaccharide-arabinoxylan
GI	Gastrointestinal
GIT	Gastrointestinal tract
Gln	Glutamine
Gly	Glycine
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMO	Genetic Modified Organisms

GMOS	Galacto-mannan-oligosaccharide
GOS	Galactooligosaccharide
GPx	Glutathione peroxidise
GR	Glutathione reductase
GRAS	Generally-recognised-as-safe
GSH-PX	Glutathione peroxidase
(H:L)	Heterophil:lymphocyte ratio
Hb	Haemoglobin
Hct	Haemaotcrit
HDP	Host defence peptides
HK	Head kidney
HMH	Hop extract
HMO	Essential oil of oregano
HMOH	HMH + HMO
HSB	Hybrid striped bass
HSP	Heat shock protein
HUFA	Highly polyunsaturated fatty acids
ICE	IL-1β converting enzyme
IEC	Intestinal Epithelial Cells
IEL	Intra-epithelial lymphocytes
IEPC	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
iIEL	Intestinal IEL
IL	Interleukin

IMI	Intra-mammary infection
IML	Intestinal Mucosal Lymphocytes
IMO	Isomaltooligosaccharide
iNOS	Inducible nitric oxide synthase
IPEC	Porcine intestinal epithelial cells
IRAK1	IL-1 receptor-associated kinase 1
IRTA	Institut de Recerca i Tecnologia Agroalimentàries
Keap1	Kerlch-like ECH-associating protein 1
LAB	Lactic Acid Bacteria
LAM	Laminarin
LB	Locust bean
LBP	Lipopolysaccharide binding protein
LDL	Low density lipoproteins
LF	Lactoferrin
LFA	*Lactobacillus*-fermented *Artemisia princeps*
LGG	Lactobacillus rhamnosus GG
LITAF	Lipopolysaccharide-Induced TNF Factor
LN	Lymph node
LNA	Alpha-linolenic acid
LP	Lamina Propria
LPS	Lipopolysaccharide
Lys:	Lysine
Lyz	Lysozyme
MAP	Mitogen-activated protein
MAPK	MAP kinase

MBL	Mannose Binding Lectin
MCLI	Modified Clinoptilolite
MCP	Monocyte chemotactic protein
MCV	Mean corpuscular volume
Met	Metionin
Mg	Magnesium
MHA	Methionine hydroxyl analogue
MHC	Major Histocompatibility Complex
MKP	MAP kinase phosphatase
MLN	Mesenteric lymph node
MNB	Mannobiose
MNC	Mononuclear cells
Mo	Monensin
MOA	Microencapsulated organic acids
MOS	Mannanoligosaccharide
MPO	Myeloperoxidase
MR	Milk Replacer
MUC	Mucin
MyD	Myeloid Differentiation
Na	Sodium
NBT	Nitro Blue Tetrazolium
NCCRP	Non-specific cytotoxic cell receptor
NCLI	Natural Clinoptilolite
NDV	Newcastle Disease Virus
NE	Necrotic Enteritis

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_3	Ammonia
NK	Natural Killer
NO	Nitric Oxide
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NZFSA	New Zealand Food Safety Authority
OA	Organic acid
ODN	Oligodeoxynucleotide
OM	Organic matter
P	Phosphorus
pBD	Porcine β-defensin
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PCV	Packed cell volume
PCR	Polymerase chain reaction
PE	Peroxinectin
PF	Propolis flavonoid
PG	Peptidoglycan
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PHB-HV	Poly-b hydroxybutyrate-hydroxyvalerate
Phy	Phytase
PIE	Porcine intestinal epitheliocyte
PKA	Potential killing activity

PLA	Phenyllactic acid
pLF	Porcine Lactoferrin
PMN	Polymorphonuclear leucocytes
PN	Phytonutrient
PO	Phenoloxidase
POD	Peroxidase
PP	Peyer's patches
PPAR	Peroxisome proliferator-activated receptor
proPO	Prophenoloxidase
PRR	Pattern Recognition Receptor
PSM	Plant Secondary Metabolites
PUFA	Polyunsaturated fatty acid
PW	Poke weed mitogen
qRT-PCR	Quantitative real time PCR
RB	Respiratory burst
RBA	Respiratory burst activity
RBC	Red blood cell
RBCC	Red blood cell catalase
RcEE	*R. coreanus* ethanolic extract
REG3G	Regenerating islet-derived 3 gamma
REMR	Relative Metabolic Rate
RFLP	Restriction fragment length polymorphism
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
RNA	Ribonucleic acid
RORC	RAR-related orphan receptor C

ROS	Reactive oxygen species
RSRP	Rabbit sacculus rotundus antimicrobial peptides
RV	Rotavirus
SAA	Serum amyloid A
SARA	Subacute ruminal acidosis
sAO	Sodium alginate oligosaccharides
SB	Sodium Butyrate
SCE	Sugar cane extract
SCFA	Short Chain Fatty Acid
scFOS	Short Chain FOS
SFPSE	*Sargassum fusiforme* polysaccharide extract
SGAMP	Swine Intestinal Antimicrobial Peptides
SGR	Specific Growth Rate
SDP	Spray Dried Product
SDPP	Porcine SDP
SE	*Salmonella enteritidis*
Se	Selenium
SFTPD	Surfactant Protein D
SID	Standardised Ileal Digestibility
sIg	Serum Immunoglobulin
SIGIRR	Single-immunoglobulin interleukin-1 receptor-related
SOD	Superoxide Dismutase
SP	Selenium Enriched Probiotics
SRB	Stabilized Rice Bran
SRBC	Sheep Red Blood Cell

SS	Sodium Selenite
SWE	Seaweed Extracts
TAB1	TGF-β Activated Kinase 1
T-AOC	Total Antioxidant Content
TBC	Total Bacterial Count
TCC	Total Coelomocytes Counts
TCR	T-Cell Receptor
TGEV	Transmissible Gastroenteritis Coronavirus
TGF	Transforming Growth Factor
Th	T helper
THC	Total haemocyte count
TLR	Toll-Like Receptor
TLI	Total lipids
TNF	Tumour Necrosis Factor
TNFSF15	Tumour necrosis factor ligand superfamily member 15
T-NOS	Total nitric oxide synthase
Tollip	Toll Interacting Protein
TOR	Target of rapamycin
TOS	Transgalactooligosaccharide
TP	Total protein
TRAF6	TNF receptor-associated factor 6
Trp	Tryptophan
TTA	Tetradecylthioacetic acid
UCP	Uncoupling protein
UMP	Uridine monophosphatase

US EPA	United States Environmental Protection Agency
US FDA	United States Food and Drug Administration
VE	Vitamin E
VEGF	Vascular endothelial growth factor
VFA	Volatile Fatty Acids
VH	Villus height
VH:CD	Villous height / Crypt depth ratio
WB	Wheat bran
WBC	White Blood Cell
WC1	Workshop cluster 1
WG	Weight Gain
WoS	Web of Science Core Collection
WSSV	White Spot Syndrome Virus
XOS	Xylooligosaccharide
YCW	Yeast Cell Wall
YDM	Yeast-derived macromolecules
YP	Yeast derived Protein
Zn	Zinc
ZnCP	Zinc-bearing clinoptilolite
ZnO	Zinc oxide
5- ALA	5-Aminolevulinic acid