

SCIENTIFIC / TECHNICAL REPORT submitted to EFSA

Bibliographic review on the potential of microorganisms, microbial products and enzymes to induce respiratory sensitization¹

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Abstract

The immune system has evolved to protect individuals from microbial pathogens as well as larger parasites. However, the immune system can sometimes react inappropriately to innocuous antigens, triggering allergic reactions. The potential of microorganisms, microbial products and enzymes to induce respiratory sensitization when used as food and feed additives was investigated in this report. A short review of the state-of-the-art methods to predict allergenicity was also conducted. Our results indicate that there is currently no established model to predict the allergenicity of a molecule. Although *in-silico* models can be useful to predict cross-reactivity between allergens, they do not take into account phenomena like the context of presentation of the antigen to the immune system. There is no reliable, predictive *in-vitro* or *in-vivo* model of allergenicity. Cases of occupational allergy to both fungi and bacteria have been documented, but allergic reactions to microorganisms purposely introduced in the work environment seem to concern only a limited number of fungi. Enzymes were more a matter of concern, with 17 out of 71 enzymes investigated in this report being linked to respiratory allergies. Because these risks are well known, enzyme exposures are strictly controlled both by regulatory authorities and

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companies. The patterns of prevalence of allergic reactions to enzyme indicate that they are more common at the level of enzyme manufacturers and large-scale users than in the general population.

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Background

The Regulation (EC) No 429/2008 concerning feed additive applications in Europe is based on a safety opinion made by EFSA (European Food Safety Authority). The Regulation includes an Annex II, which provides the general requirements that should be satisfied by a dossier supporting an application for the use of a product as a feed additive. In particular Section III of the Annex II deals with safety of a feed containing an additive, and this is further subdivided into safety for target animals (section 3.1), for consumers of the products derived from the animals fed the additives (3.2), for users/workers exposed to the additive (3.3), and finally for the environment (3.4). In section 3.3, it is stressed that the allergenic potential and sensitization via the respiratory route is of concern, and should be evaluated. Annex III of that Regulation provides the specific requirements concerning specific intended additives depending on the technical nature of the feed additive. In this context, technological additives and zootechnical additives are of interest. Thus, in spite of the guidance on toxicological safety component of a feed additive dossier [1], EFSA is instructing that enzymes and microorganism should be assumed *a priori* to be respiratory sensitizers unless convincing evidence to the contrary is provided.

In order to investigate the potential of microorganisms, microbial products and enzymes to induce respiratory sensitization when used as food and feed additives, we conducted a systematic literature search of all the information we could find linking microorganisms and enzymes to respiratory sensitization. We supplemented this search with references from important reviews in the field of enzymes and microorganisms and occupational health, as well as with interviews with some leading companies in these fields. Finally, we conducted a survey of existing regulations on the subject in developed countries.

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1. Introduction

1.1. The hypersensitive diseases and their pathophysiological background

1.1.1. Type I hypersensitivity: Allergy

The immune system has evolved to protect individuals from microbial pathogens as well as larger parasites. However, the immune system can sometimes react inappropriately to innocuous antigens. Allergic reactions occur when an individual produces IgE antibody against such innocuous antigens, or allergens.

While other antibodies are free circulating in blood or extracellular fluid, IgE is also found in tissues, where it is tightly bound to the mast-cell surface through the IgE receptor. Exposition to the allergen triggers a cascade of responses initiated by the activation of mast cells by IgE-receptor cross-linking [2]. Basophils and activated eosinophils can also act in a similar fashion. IgE is thought to have evolved as a mechanism to fight off large multicellular parasites, like nematodes and trematodes, less vulnerable to other effector arms of the immune system (like phagocytosis or CD8 T cells) [3]. Indeed, the typical symptoms of type I hypersensitivity, like smooth muscle contraction, itching and generation of mucus, seem appropriate to dislodge such organisms. However, modern hygiene practices have considerably lessened the population's exposure to parasites in developed countries, and the focus on IgE is overwhelmingly set on its role in the pathophysiology of allergy. It is estimated that almost half the populations of North America and Europe have allergies to one or more common environmental antigens [4].

Why some antigens lead to IgE production and type I hypersensitivity is not fully elucidated. Certain antigens and routes of antigen presentation to the immune system are known to elicit preferably a Th2 response characterized by the production of IgE. Most allergens are relatively small, highly soluble proteins. The typical presentation of an inhalable allergen involves a transmucosal route and very low doses (as low as 1 microgram per year for some pollen allergies) [5]. Myeloid dendritic cells are the dominant antigen-presenting cells in the respiratory mucosa, and they are known to be very efficient Th2 inducers. Exposure to low doses of antigen in general also tends to favor a Th2 response in the absence of Th1-inducing co-stimulatory signals.

Unlike pathogens that are usually eliciting the same pathology in a large majority of the population (albeit with difference symptoms or severity), only some of the people who are exposed to common allergens make IgE antibodies against them. In addition, there is a clear trend of increase in prevalence of atopic allergy, and of asthma in particular, in developed countries. Environmental factors that may potentially explain these trends are changes in

exposure to microorganisms in early childhood, environmental pollution, allergen levels, and dietary changes [6].

Alterations in exposure to microorganisms are the most plausible explanation at present for the increase in atopic allergy. This includes exposure to pathogens, but also to a large number of commensal, non-pathogenic microorganisms early in life [7]. Epidemiologically, atopy is negatively associated with a history of infection with measles or hepatitis A virus, and exposure to *Mycobacterium tuberculosis*, but severe respiratory syncytial virus (RSV) infection in children is positively correlated to the later development of asthma. The underlying idea behind this theory would be that Th1 immune response early in life might reduce the likelihood of Th2 responses later in life and vice versa. More recent versions of this theory focus on the role of Tregs as opposed to inflammatory Th1 [8]. Broad exposure to commensal bacteria early in life is associated with protection against IgE-mediated hypersensitivity.

The relationship between environmental pollution and type I hypersensitivity is unclear [4]. Some epidemiological studies have shown a lower prevalence of allergy and atopy in severely polluted cities than in generally cleaner ones. Other studies on diesel-fuel particulates show that they trigger symptoms in patients. Studies of the effects of exposure to secondhand tobacco smoke or occupational hazards on atopic sensitization have also led to discordant results. Similarly, there is no clear evidence that the rising prevalence of allergy is due to any systematic change in allergen exposure or to changes in diet.

The most common route of allergen exposure is inhalation. Most often, it initially leads to mild allergies causing allergic rhinitis, with sneezing and a runny nose as main symptoms. This results from the activation of mucosal mast cells beneath the nasal epithelium by allergens such as the proteins from pollens [9]. Allergic rhinitis is characterized by intense itching and sneezing, local oedema leading to blocked nasal passages, a nasal discharge, and irritation of the nose as a result of histamine release. Allergic conjunctivitis is a similar condition triggered by airborne allergens deposited on the conjunctiva. These reactions are annoying but cause little lasting damage when they are linked to seasonal allergens like pollen.

More severe damage can come from activation of mast cells in the lower airways, which leads to allergic asthma [10]. It is a chronic condition that requires treatment and can be life-threatening. Both allergic asthma and rhinitis can come from constant exposure to occupational allergens as it is the case for fungal enzymes and baker's asthma, but asthma can also be perpetuated even in the absence of further exposure to allergen. The airways become characteristically hyperreactive and factors other than reexposure to antigen can trigger asthma attacks. It is worth noting that, although asthma has potentially more severe consequences, quality of life studies indicate that rhinoconjunctivitis is perceived as worse

than bronchial asthma for the patient [11]. This is probably linked to the fact that asthma is treated more efficiently and more strictly than rhinoconjunctivitis.

1.1.2. Type III hypersensitivity: immune complex disease

Type III hypersensitivity reactions are caused by inhalation of soluble environmental antigens that trigger immune responses by precipitated antibodies (mainly IgG) and lymphocytes; the characteristics of hypersensitivity pneumonitis (HP) are summarized from recent reviews [12,13,14,15,16]. Unlike allergic reactions, they are linked to IgG rather than IgE production. The pathology is caused by large aggregates of IgG:antigen complexes, also known as immune complexes. These immune complexes fix complement and can bind to and activate leukocytes bearing Fc and complement receptors; these in turn cause widespread tissue injury to small blood vessels in many tissues and organs, including the skin, kidneys, and nerves. This is thought to be the underlying pathophysiological mechanism in hypersensitivity pneumonitis (also called allergic alveolitis). Immune complexes also form in autoimmune diseases such as systemic lupus erythematosus where, because the antigen persists, the deposition of immune complexes continues, and serious disease can result.

HP has been described in various settings, often involving organic particles and environments where moulds abound: farming, bird-breeding for example. Some chemical compounds have also been shown to be able to act as haptens and cross-link the host albumin to create an allergic particle. Common industrial antigens causing HP include isocyanates (paint sprays), plastics (packing plants), *Mycobacterium avium* complex (metal working fluids), *Aspergillus* (agriculture), and thermophilic actinomyces (agriculture). Genetic factors are important for development of HP and HP occurs seldom in smokers.

The most likely reason why some inhaled allergens provoke IgG rather than IgE is probably linked to doses of exposure. Occupational allergens associated with HP are generally linked with very high levels of exposure, such as in the case of metalworking fluids or farmer's lung.

HP can present acute, subacute or chronic forms. The chronic form, with lung fibrosis, can be very severe, and even life-threatening. The 5-year mortality in chronic HP is as high as 30%, and in the subset of the fibrotic type of chronic HP, the 5-year mortality reaches 61%. In the acute form, respiratory symptoms are lung crackles, cough, dyspnoea, fever, myalgia, headache, and malaise. If the exposure continues, the acute form develops into the subacute (<4 month of exposure) and further into the chronic form (> 4 month of exposure). During the continued exposure, cough and dyspnoea at exertion gradually become worse. In the acute form, high resolution computed tomography (CT) shows ground-glass lung opacities and micronodules that may progress with addition of air-trapping, emphysema and fibrosis in the chronic form. Pulmonary function tests show low carbon monoxide diffusion capacity and typically a restrictive pattern.

1.2. Respiratory hypersensitivity to microorganisms in the working environment

1.2.1. IgE-mediated responses

In spite of spores from naturally occurring fungi being described as a common cause of asthma and allergies, there is little description of occupationally induced fungal allergies.

1.2.2. Hypersensitivity pneumonitis

Microorganisms have been involved in numerous outbreaks of HP in industry, agricultural work and in indoor climate; examples of involved organisms are listed in Table 1. Several outbreaks of HP have occurred due to aerosol exposure to water-based metalworking fluids contaminated with microorganisms. In several of these cases, *Mycobacterium* species were considered the offending agents. Mycobacteria are ubiquitous in the human environment, including in natural water, drinking water and soil. They are easily aerosolized due to their hydrophobic surface. Growth of mycobacteria may have been promoted in metalworking fluids by the use of biocides and disinfectants that have diminished or eradicated competing microorganisms; mycobacteria are highly resistant to quaternary ammonium compounds, formaldehyde, morpholine, chlorine, chloramines, ozone and heavy metals [17,18,19].

HP in farmers caused by exposure to mouldy livestock feed is known as farmer's lungs, which is associated with exposures to thermophilic actinomycetes and fungi [20,21]. Workers engaged in packing of peat moss, an organic substance derived from sphagnum moss, have also developed HP from contamination by moulds. The peat moss contained high levels ($\sim 5 \times 10^7$ colony forming units/g) of *Monocillium* species and *Penicillium citreonigrum* [22]. Sphagnum vegetation may also contain high levels of mycobacteria as *M. avium* and *M. intracellulare* [18].

HP may be due to microorganisms playing an intended role in production processes. HP in workers cultivating edible mushrooms, mushroom worker's lung, may be due to microorganisms [20,23,24]. Thus, mushrooms are cultivated on compost that can contain high amounts of thermophilic actinomycetes and moulds, having a role in the production of the compost. The actinomycetal spores can be found in high levels in the air during preparation of the compost, during the spawning ($\sim 10^9$ colony forming units/m³), i.e. inoculation with mushroom mycelium, or during other work in the mushroom house [24]. Additionally, mushroom worker's lung may be due to the spores from exotic mushrooms [20,23,25]. However, no sensitization was observed to the white button mushroom *Agaricus bisporus* in a

study in patients with mushroom worker's lung. Use of airway protection during spawning prevented respiratory complaints [24].

Cheese workers' lung

Mushroom worker's lung and cheese worker's lung are among the types of HP where the microorganisms have a role in the production processes. Although not commonly encountered, HP may be caused by unintentional growth of moulds and bacteria in buildings with excessive inside moisture. *Aspergillus niger* is a common antigen accounting for development of serum precipitins and HP. *Stachybotrys* species are commonly found in damp buildings with cellulose material, but it has not been implicated as a cause of HP [26]. Inhalation of trichosporon yeasts, which have contaminated home environments during hot and humid summer-seasons are a common cause of "summer-type" HP in Japan [27,28]. Furthermore, different strains of mycobacteria have been isolated from mouldy buildings [19].

Overall, in cases where HP appeared due to unintended exposures to microorganisms, exposures have often been related to wet or humid conditions that promoted the growth and thus, resulted in unusually high exposures. Also in these cases, the work conditions may have promoted aerosol formation. Thus, primary prevention should, where possible, limit wet or humid conditions or otherwise, growth should be controlled. If high exposures cannot be avoided, respiratory protection has to be worn. Aerosol formation should always be minimized. Where microorganisms have intended roles in a production, process specific risk management have to be introduced. Both for unintended exposures and for exposures to intentionally used microorganisms, spore-producing microorganisms are often the cause of HP.

Table 1. Hypersensitivity pneumonitis due to exposure to microorganisms

Exposure conditions	Agent	Reference
Exposures to aerosols of contaminated water-based metalworking fluid	Acid-fast mycobacteria, including <i>M. chelonae</i> , <i>M. immunogenum</i> and <i>M. abscessus</i> . Gram-positive bacteria as <i>Rhodococcus sp.</i> , <i>Bacillus sp.</i> , incl. <i>B. cereus</i> , <i>Staphylococcus sp.</i> , <i>Actionomycetes sp.</i> , <i>Acinetobacter haemolyticus</i> , <i>Micrococcus sp.</i> , <i>Corynebacterium nitrilophilus</i> .	[29], [30], [31], [32]

	<p>Gram-negative bacteria as <i>Pseudomonas</i>, <i>Acinetobacter</i>, <i>Deleya aesta</i> and <i>Ochrobactrum anthropi</i></p> <p>Fungi as <i>Aspergillus niger</i>, <i>Fusarium sp.</i>, <i>Acremonium sp.</i>, <i>Candida stelatoides</i>, <i>Geotrichum</i> and <i>Rhinochadiella</i></p>	
<p>Exposure to mouldy livestock feed, which can cause "farmer's lung", and mouldy compost</p>	<p>Thermophilic actinomycetes as <i>Faenia retivirgula</i> (<i>Saccharopolyspora rectivirgula</i>), <i>Saccharomonospora viridis</i> and other thermophilic species as <i>Thermoactinomyces vulgaris</i> and <i>Thermoactinomyces sacchari</i>.</p> <p>Fungi as <i>Aspergillus sp.</i>, for example <i>A. fumigatus</i>, <i>A. flavus</i> and <i>A. umbrosus</i>, <i>Penicillium sp.</i> and <i>Cladosporium</i></p>	[21], [20], [33]
<p>Cultivating, picking and packing of commercial mushroom crops may cause "mushroom workers' lung" due to microorganisms and mushroom spores</p>	<p>Thermophilic actinomycetes as <i>Excellospora flexuosa</i>, <i>Thermomonospora alba</i>, <i>Thermomonospora curvata</i>, <i>Thermomonospora fusca</i>, <i>Micropolyspora faei</i> and, <i>Thermoactinomyces vulgaris</i></p> <p>Fungi as <i>Cladosporium sphaerospermum</i>, <i>Penicillium frequentans</i> and <i>Scopulariopsis sp.</i></p> <p>Mushroom spores from several species also produced HP</p>	[24], [20], [25], [23]
<p>Buildings with excessive inside moisture</p> <p>Summer-type HP in Japan</p>	<p>Moulds as <i>Aspergillus niger</i></p> <p>Yeast as <i>Trichosporon</i> species</p>	[26], [27], [28]

1.3. Respiratory hypersensitivity to enzymes in the working environment

1.3.1. IgE-mediated responses

Experiences with enzymes in the detergent industry unequivocally demonstrated exposure-dependent effects of sensitization and development of allergies, mainly rhinitis, conjunctivitis and asthma. Thus, the introduction of alkaline and heat-stable proteolytic enzymes into the detergent products in the 1960s led to high exposures (in the $\mu\text{g}/\text{m}^3$ range) and sensitization of 50-70% of the workers, with nearly 20% of these individuals suffering from occupational allergies and asthma [34,35]. In addition to proteases, other enzymes such as amylases, lipases and cellulases are commonly used by the industry [35].

Strict exposure control programs reduced the exposures to low levels ($\leq 15 \text{ ng}/\text{m}^3$ range), which in turn reduced sensitization and prevented the onset of allergic symptoms [34,35]. This low exposure level has been set by taking into account the fact that the detergent matrix behaves as an immunological adjuvant [35]. Another important finding was that induced sensitization was observed at exposure concentrations that were lower than concentrations needed to elicit enzyme induced allergic symptoms [34,35].

In a case-reference analysis of a cohort of employees in a European detergent factory, chest disease was not increased significantly at $\leq 3.9 \text{ ng}/\text{m}^3$ of proteases and eye and nose symptoms were not increased at $\leq 2.3 \text{ ng}/\text{m}^3$. The authors mention that only the protease level was measured although amylases and cellulose were also used, and that “irritant” dust and non-occupational reactions may have contributed to the findings above these levels [36].

Outbreaks of sensitization, upper airway symptoms and asthma still occurred in the detergent industry where strict exposure controls were not followed. In such a case, the protease level was in the range from 4 to $57 \text{ ng}/\text{m}^3$, several enzymes were used and amylase was found to be a more potent sensitizer than the protease [37].

Allergic reactions were observed in Swedish consumers in the early period of use of enzymes in detergents, when products were dusty and enzymes were not encapsulated. A re-creation using the product and the consumer habits at the time suggested that exposure may have been as high as $200 \text{ ng}/\text{m}^3$ [35]. Recently, a study was performed in 581 atopic Filipinos who had a daily use of hand laundry for several hours; the study lasted up to 2 years. The laundry contained a protease and an amylase. The laundering was with granular products or with bar products that were also used for personal cleansing. Exposures were low ($\leq 0.18 \text{ ng}/\text{m}^3$) for the granular product, lower ($\leq 0.026 \text{ ng}/\text{m}^3$) for the bar product for scrubbing, and below the detection limit ($< 0.007 \text{ ng}/\text{m}^3$) for personal cleansing. None of the subjects developed

enzyme specific IgE antibodies, and the authors suggested that a threshold exists for sensitization to detergent enzymes [38].

Long-term studies did not show a consistent trend in accelerated decrease in lung function due to enzyme exposure [39,40,41]. This suggests that allergies, especially asthma, are the major risk factor due to enzyme exposures in the detergent industry. Overall, these findings support that a “practical” NOAEL for enzymes may be in the low ng/m³ range, with specific levels for each specific enzyme. Also, enzyme exposures in the detergent industry have been controlled to a few ng/m³ [35,36].

1.3.2. Hypersensitivity pneumonitis

In contrast to allergic airway diseases, HP to proteolytic enzymes is seldom reported in the medical literature, although case reports do exist. Thus, a 53-year-old woman developed HP due to washing surgical instruments as well as cleaning operating room surfaces with a subtilisin containing cleaner. She had quit smoking 10 years previously. She wore latex gloves and a mask (not specified) for protection during work [42]. A 43-year-old male developed HP due to phytase; he had been a non-smoker for 20 years. He worked in a cattle feed factory, where his job was to take samples of the cattle feed for quality control. Aerosolized phytase was added to the feed in a closed system. When taking samples, the patient did not wear airway protection. On the floor where phytase was added, the airborne concentration was 8.7-38.4 µg/m³, whereas the concentrations at other floors were 6-58 ng/m³. No antigen specific IgE was detected in patient’s serum, but the level of specific IgG was high [43]. In this case HP was due to a very high exposure to phytase. Allergy due to phytase has been reported in several studies as discussed in a recent review [44]. In the detergent industry where a substantial number of IgE mediated allergies have appeared, enzyme exposures have not been associated with HP or emphysema (c.f.[35]).

Overall, taking into account the high number of cases of allergy in relation to exposure to enzymes and the low number of cases with HP, the type I allergy is considered the critical effect and it is inferred that protection against allergic airway diseases will also protect against HP.

2. Objectives

Identification of key parameters:

microorganisms (the relevant types, forms and species are organisms that are known to be used per se in feed and food)

enzymes that are used in feeds and food

sensitization (main emphasis on IgE-related respiratory sensitization but also allergic alveolitis, and non-immunological hypersensitivity are covered). Physiological/immunological mechanisms, distinction between the exposed organ and the target organ/symptoms (i.e. can respiratory sensitization occur via other exposures?) are discussed.

It is important to emphasize the ubiquitous occurrence of microorganisms, their products and enzymes in all biological materials versus the deliberate addition in feed or food. In the allergen research of today many allergens from plants and animal sources are cloned and expressed in *E. coli* or *Pichia* for research purposes. Such applications are excluded (but documented) at an early stage of the literature search.

To which extent are microorganisms, microbial products and enzymes respiratory sensitizers?

To solve this problem three parallel approaches have been taken:

- An epidemiological search. The literature on epidemiological data related to respiratory allergies and sensitization will be screened in order to identify descriptions of reactions to microorganisms, microbial products and enzymes.
- A clinical/case based search. Case stories and descriptions of isolated outbreaks of respiratory sensitizations and allergies will be documented
- An allergen based search. Literature will be searched for occurrences of microorganisms, their products or enzymes.

Currently available methods to test respiratory sensitization and their validity

- Structure-allergenicity-relationships (prediction of allergenicity based on the primary-secondary-tertiary protein structure)
- *In vitro*-models for antigen presentation and sensitization.
- Animal models of allergic sensitization

3. Materials and Methods

3.1. Definition of the specific biological ingredients in foods & feeds

Microorganism: microbiological entity, cellular or non-cellular, capable of replication and/or of transferring genetic material. Includes fungi and bacteria (also under the form of spores). Viruses, archea, protists, as well as microscopic plants and animals are generally also included in this definition, but have little relevance for this report, as they are not used as food or feed additives.

Microbial product: pool of organic compounds that are released from biomass growth and/or decay. Includes (but is not limited to): humic and fulvic acids, polysaccharides, proteins, nucleic acids, organic acids, amino acids, antibiotics, steroids, exocellular enzymes, siderophores, structural components of cells and products of energy metabolism.

Enzyme: enzymes are proteins from animal, vegetal or microbial origin that catalyze chemical reactions. Relevant enzymes for this report include (but are not limited to): amylases, proteases, trypsin, cellulases, pectinases, xylanases, papain, and lactases.

It is agreed upon that this report will focus exclusively on microorganisms, microbial products and enzymes that are added deliberately to food and feed. Additionally, those additives need to have been clearly and individually defined to be taken into consideration.

The lists of microorganisms and enzymes (Appendices 1 and 2) were compiled by using the sources listed in table 2:

Table 2: sources for the list of microorganisms and enzymes considered in this report

	Food	Feed
Microorganisms	QPS/Danish FDA	Community register of feed additives/QPS
Enzymes	AMFEP	Community register of feed additives/AMFEP

3.2. Systematic Pubmed search

In order to narrow down our search, we established a list of relevant microorganisms and enzymes of interest. It was agreed that the focus of the report would be on IgE-related sensitization, as well as allergic alveolitis and non-immunological hypersensitivity. For each item on these lists, the following Pubmed search was conducted:

Item AND (IgE OR hypersensitivity OR rhinitis OR conjunctivitis OR asthma OR urticaria OR anaphylaxis OR allergic alveolitis)

When the outcome of the search was 0 hit, the item was also searched in the allergome database. When the outcome was between 1 and 500 hits, the titles of hits were screened manually to identify articles susceptible to contain epidemiological, clinical or laboratory data. Reviews were systematically included. After the title screening, abstracts were then viewed to determine which articles were relevant. When the outcome was larger than 500 hits, the search was narrowed using the following strategies, in that order:

use **“item”** instead of **item**

include only hits containing the item in abstract/title

include only hits containing the item in title

3.3. Additional literature

We used some of the most authoritative reviews in the field of respiratory sensitization and/or industrial use of enzymes and microorganisms to backtrack through ISI's Web of Knowledge the most important articles that they referenced.

3.4. Legislation and regulations

Our subcontractor (Gunnar Nielsen) made a survey of existing legislation and regulations concerning enzymes and microorganisms in developed countries.

3.5. Interview with the industry

In order to enhance our understanding of the relationship between food and feed additives and respiratory sensitization, we interviewed representatives of two of the leading companies in the field: Chr. Hansen A/S and Novozymes. The details on these companies are listed in Annex 4. These companies were first mailed a list of our questions (listed in Annex 4), after which they agreed to meet us, and in the case of Chr. Hansen A/S, to show us one of their production lines.

4. Results

4.1. State-of-the-art of the test methods

4.1.1. *In-silico* testing

The allergenicity of a protein can refer to two properties: either its capacity to induce IgE thru an immune response or its capacity to cross-react with IgE induced by a previous antigen [45]. The first case involves interaction with and processing by antigen-presenting cells, but also contacts with epithelial cells that can modify the Th1/Th2/Treg balance towards a given antigen. It is overall a very complex matter, and large parts of the theory behind it remain unexplained. It is therefore difficult to predict, and *in-silico* attempts to model this have been largely unsuccessful, although numerous hypothesis have been advanced. No common structural feature between allergens can be conclusively pointed at. The only common properties of allergens so far are that they can evade Th2-suppressing mechanisms and interact with dendritic and mast cells.

Current allergen prediction algorithms are better suited to predict IgE cross-reactivity. Past algorithms have focused on the association of unknown sequence with a single allergen motif or an allergen peptide [46]. However, recent developments are now taking into account the fact that allergens generally contain multiple epitopes [47], that these epitopes are very often discontinuous [48], and that antibodies have 2 interaction sites to a given antigen [49]. Taken together, these facts have led to algorithms focused on several motifs, with a marked improvement in false positive rates [50].

4.1.2. *In-vitro* models for antigen presentation and sensitization

As of today, *in-vitro* models are used to investigate the mechanisms of allergic diseases and the processes of each individual type of cell, rather than the allergenicity of a product. Fundamental knowledge is still missing to create an *in-vitro* model of the various cell interactions that could mimic the human response to an allergen. There is, to our knowledge, no reliable, established *in-vitro* way to predict allergenicity. In recent years, the UE has supported a large project called Sens-it-iv (<http://www.sens-it-iv.eu/>), whose goal is to develop such an *in-vitro* model. In 2009, the members from the project published 22 scientific articles on the topic. Although promising, this research has not yet reached its goal.

Respiratory sensitization and the symptoms that it causes require a complex interaction between several cell types, including epithelial cells, dendritic cells, macrophages, CD4+ T cells, B-cells and mast cells. The variety of *in-vivo* models used for respiratory sensitization

has been thoroughly reviewed by Verstraelen *et al* [51], and this section only briefly describes their findings.

Three main types of epithelial cells have been used as models: primary airway epithelial cells, alveolar cells lines, and bronchial cell lines. Primary cells are of course the closest representation of the airway epithelium, but their scarcity and donor variation is a major problem for the reproducibility of the experiments.

Alveolar macrophages studies often use cells collected by bronchoalveolar lavages for *ex-vivo* studies. Several monocytic cell lines also exist, but like with all cell lines, whether they accurately reflect the behavior of functional primary cells that they resemble is subject to discussion.

Monocyte-derived dendritic cells (DCs) have replaced Langerhans cells as primary DCs for *in-vitro* experiments. However, some very satisfying cell lines are also available, such as MUTZ-3, which display all the antigen processing and presentation pathways from a functional DC.

Due to the importance of interaction and co-stimulation by different cell types, co-culture models have been subject of much interest. Successful tri-cultures (macrophages/DC/epithelial cells) show great promise. 3D cultures, although in need of optimization, are also very interesting alternatives.

4.1.3. Animal models of allergic sensitization

Currently, the most widely used experimental animal for modelling allergic responses in the airways is the mouse. Numerous antigens can be used for sensitization, such as ovalbumin, dust mite and *A. fumigatus*. Typical induction of asthma in murine models has 2 requirements: a systematic immunisation to trigger Th2 immunity and a repeated pulmonary allergen challenge. Pulmonary challenge only without systemic immunity is not sufficient to elicit pulmonary symptoms [52,53]. The intraperitoneal route is the most common way to elicit Th2 immunity, although repeated instillation in the upper airway can also be used for some antigens. For example, Johnson *et al.* developed a house dust mite model where sensitization takes place *via* the airway [54]. Interestingly allergic asthma in mice does not require an allergenic antigen [55]. This observation is in stark contrast with the observations in humans: since patients tend to be allergic to similar allergens, allergenic properties of the antigen are deemed important. On top of these considerations, most models use BALB/c mice due to their high IgE-responder phenotype.

Although the mouse model is the most popular in the field of allergy research, it suffers from several flaws that limitate its predictive value. The differences in induction mechanisms, together with physiological and immunological differences between mice and humans might make it more suitable for clinically-oriented studies than for allergenicity prediction. Relevant models to study allergenic properties would need to rely on sensitization from inhalation only,

and these are rare. Interestingly, our discussion with researchers from Novozymes A/S is corroborating this line of thought: animal models are out of favor in their labs, and remain only used for relative allergenicity experiments. It is worthwhile to note that in-house research at Novozymes A/S has failed to show significant differences in relative allergenicity between different enzymes. This may be due to the fact that physical properties (size, formulation) matter more than sequences and functions, but it could also be another indication of the poor predictability of the mouse model.

Table 3 summarizes the various models currently used in respiratory allergy research. Models of non-respiratory endpoints such as allergic contact dermatitis e.g. the guinea pig maximization test are not included:

Table 3: Models used in respiratory allergy research

	Model	Endpoints	Pros	Cons
<i>In-silico</i>	Prediction of de-novo sensitization	Similarity of structure		Does not take into account the context of allergen presentation to the immune system
<i>In-silico</i>	Cross-reactivity to pre-existing IgE	Similarity of structure	New algorithms take into account multiple, discontinuous epitopes	Post-translational modifications are difficult to incorporate to the model
<i>In-vitro</i>	Primary epithelial cells	Molecular mechanisms within the cell (receptor activation, cytokine and chemokine production, genomics,...)	Closest model to normal epithelial cells	Used to investigate the role of epithelial cells in allergic diseases. NOT a predictive model
<i>In-vitro</i>	Epithelial cell lines	Molecular mechanisms	Homogeneity of the model,	Modified

		within the cell (receptor activation, cytokine and chemokine production, genomics,...)	reproducible results	phenotypes Used to investigate the role of epithelial cells in allergic diseases. NOT a predictive model
<i>In-vitro</i>	Primary macrophages from bronchalveolar lavages	Molecular mechanisms within the cell (receptor activation, cytokine and chemokine production, genomics,...)	Closest model to normal macrophages	Used to investigate the role of macrophages in allergic diseases. NOT a predictive model
<i>In-vitro</i>	Macrophages (cell lines)	Molecular mechanisms within the cell (receptor activation, cytokine and chemokine production, genomics,...)	Homogeneity of the model, reproducible results	Modified phenotypes Used to investigate the role of macrophages in allergic diseases. NOT a predictive model
<i>In-vitro</i>	Monocyte-derived dendritic cells	Molecular mechanisms within the cell (receptor activation, cytokine and chemokine production, genomics,...)	Close to normal DC	Heterogenous model Used to investigate the role of dendritic cells in allergic diseases. NOT a predictive model

<i>In-vitro</i>	Dendritic cell lines	Molecular mechanisms within the cell (receptor activation, cytokine and chemokine production, genomics,...)	Homogenous model Displays all characteristics of normal DCs	Used to investigate the role of dendritic cells in allergic diseases. NOT a predictive model
<i>In-vitro</i>	Three dimensional co-cultures	Molecular mechanisms within the cell (receptor activation, cytokine and chemokine production, genomics,...)	Takes into account the multiple cells lines involved in the response to allergens	Far from being optimized
<i>In-vivo</i>	BALB/c mice, OVA +/- adjuvant, intraperitoneal injection followed by multiple respiratory challenges	IgE and IgG levels T-cell proliferation Respiratory function	Well-developed model	Used to investigate pathological mechanisms Does not mimic human exposure to allergen “Atopic-like” mouse NOT a predictive model
<i>In-vivo</i>	BALB/c mice, +/- adjuvant, respiratory sensitization	IgE and IgG level T cell proliferation Respiratory function	Mimics the exposure in humans	Limited to a few allergens so far (OVA, birch pollen)
<i>In-vivo</i>	BDF1 mice, Japanese Cedar pollen +	IgE and IgG	Mimics the exposure in	Limited to a few

	adjuvant, respiratory sensitization	levels	humans	allergens
<i>In-vivo</i>	Guinea pig	Clinical signs	Oldest model Good asthma model	Requires pre-treatment with anti-histamines to modulate the immune response
<i>In-vivo</i>	Rat, systemic sensitization followed by multiple respiratory challenges	IgE Systemic and airway markers of inflammation	Larger size than mice Good to understand tolerance mechanisms	Develops tolerance upon repeated challenges NOT a predictive model
<i>In-vivo</i>	Dog, asthma model	IgE Clinical symptoms (rhinitis, conjunctivitis)	Naturally develop allergies to human-relevant allergens	Extremely labour intensive and expensive
<i>In-vivo</i>	Sheep, asthma model	IgE Clinical signs	Variability in responders to a given allergen, like in humans	Cost, labour Different response to platelet activated factor pathway than humans

4.2. Cultures of microorganisms allowed in foods & feeds

This section describes the results of the systematic literature search that was conducted on Pubmed, together with the extra information that we found by back-tracking references from important reviews through Web of Knowledge. For each microorganism or microbial product from our list, we listed all the relevant information we could find in the following order: **microorganism,**

use in food or feed (examples of use of the microorganism in food or feed, non-exhaustive)

epidemiological studies

case reports

literature arguing against possible sensitization by the agent.

28 species were found to have relevant information, for a total of 57 relevant articles. Only species for which relevant information could be found are listed here.

Arthrobacter globiformis

Use in food or feed: *A. globiformis* is used to alter the taste of some food products, for example to metabolize bitter limonin of citrus.

Epidemiological studies: a study of 20 agricultural workers from Poland diagnosed with hypersensitivity pneumonitis showed that *A. globiformis* was the etiological agent in 8 cases [56].

Case reports: n/a

Literature arguing against a possible sensitization by the agent: n/a

Aspergillus niger

Use in food or feed: Various strains of the fungus *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574). *A. niger* also is used to produce enzymes such as glucoamylase and pectinases.

Epidemiological studies: n/a

Case reports: a case of hypersensitivity pneumonitis to *A. niger* was reported in 1996 [57], while several cases of allergic fungal sinusitis, rhinitis and bronchopneumonitis have been reported [58,59,60]. It has been suggested that asthma and atopy may both represent a protective response against contained airway infection due to ubiquitous proteinase-producing fungi [61].

Literature arguing against a possible sensitization by the agent: n/a

Aspergillus oryzae

Use in food or feed: *A. oryzae* is a fungus used in Chinese and Japanese cuisine to ferment soybeans or to saccharify rice, other grains, and potatoes in the making of alcoholic beverages. *A. oryzae* is also used for the production of rice vinegars. It can also be used to produce glucoamylase and alpha-amylase.

Epidemiological studies: n/a

Case reports: 2 articles were found describing cases of respiratory sensitization linked to the use of *A. oryzae* in the food industry [62,63]. Both cases involved a high seasonal concentration of spores in the homes of Japanese brewers and respiratory symptoms. Interestingly, one of these articles referred to the existence of at least 5 other similar cases described in the Japanese literature.

Literature arguing against a possible sensitization by the agent: n/a

Bacillus licheniformis

Use in food or feed: *B. licheniformis* is used to degrade feathers to produce cheap and nutritious feed for livestock. Also used as a probiotic in livestock.

Epidemiological studies: n/a

Case reports: n/a

Literature arguing against a possible sensitization by the agent: *B. licheniformis* spores were found to induce a Th1 type response *in-vitro* in Bal/C mice [64].

Bacillus pumilis

Use in food or feed: *B. pumilis* is used to degrade feathers to produce cheap and nutritious feed for livestock.

Epidemiological studies: In 1995, Bernstein *et al* [65] found 6 cases of machine operator's lung, a type of hypersensitivity pneumonitis. Those cases all followed occupational exposure to metalworking fluid. At least 2 of those patients presented serum precipitin tests positive to *B. pumilis*.

Case reports: n/a

Literature arguing against a possible sensitization by the agent: n/a

Bacillus subtilis

Use in food or feed: *B. subtilis* can be used in the commercial production of the Japanese food natto as well as the similar Korean food cheonggukjang. It can also produce amylase; and is used as a probiotic for livestock.

Epidemiological studies: a cross-sectional study of 51 workers from a herb processing plant showed that they responded with a high frequency 73% to a skin prick test with extract from *Bacillus subtilis* [66].

Case reports: 6 cases of hypersensitivity pneumonitis were described in one family, caused by wood dust created by home improvement. All six patients had positive bronchoprovocation responses to vegetative cell extracts of *B. subtilis* [67].

Literature arguing against a possible sensitization by the agent: n/a

Bifidobacterium (adolescentis, animalis, animalis ssp. lactis, bifidum, breve, infantis, lactis, longum, pseudolongum, thermophilum)

Use in food or feed: The genus *bifidobacterium* contains several species that are commensals from the gut flora. They are used as probiotics in milk and dairy products, and sometimes as probiotics for livestock.

Epidemiological studies: n/a

Case reports: n/a

Literature arguing against a possible sensitization by the agent: *B. animalis* reduced several immune parameters in an animal allergy model as well as in an autoimmunity model and skewed the Th1/Th2 balance towards Th1 in females [68]. Similarly, *B. longum* suppressed Th2 responses [69], increased Foxp3 regulatory cells numbers [70] and showed potential to prevent IgE-mediated immune responses [71]; all in murine models. In a human clinical trial in Japan, *B. longum* prevented the increase of blood thymus and activation-regulated chemokine (TARC) levels during pollen season in pollinosis patients [72]. TARC level increase is an indicator of disease severity in pollinosis.

Debaryomyces hansenii

Use in food or feed: *D. hansenii* is generally the predominant yeast in the smear of bacterial surface-ripened cheeses such as Munster.

Epidemiological studies: n/a

Case reports: a 65-year-old female was reported to have developed cough, fever and dyspnoea following repeated exposure to a home ultrasonic humidifier [73]. Precipitating test and lymphocyte proliferative response was positive for an extract of *D. hansenii*.

Literature arguing against a possible sensitization by the agent: n/a

Enterococcus faecium

Use in food or feed: *E. faecium* is a probiotic used in feeds for livestock.

Epidemiological studies: n/a

Case reports: n/a

Literature arguing against a possible sensitization by the agent: in the atopic dermatitis mouse model, *E. faecium* suppressed total IgE production and induced IL-12 and IFN-gamma production; intraperitoneal injection of strain T120 inhibited serum IgE elevation and atopic dermatitis symptoms [74].

Geotrichum candidum

Use in food or feed: this organism is used to produce certain types of yoghurts, especially from Nordic countries.

Epidemiological studies: A Australian study of 14 patients with HP from domestic exposure to moulds showed 2 patients with precipitin and inhalation test positive to *G. candidum* [75].

Case reports: n/a

Literature arguing against a possible sensitization by the agent: n/a

Lactobacillus (acidophilus, alimentarius, amylolyticus, amylovorans, brevis, brevis var. lindneri, buchneri, bulgaricus, carnis, casei, casei rhamnosus, cellobiosus, collinoides, curvatus, delbrueckii, delbrueckii ssp. bulgaricus, delbrueckii ssp. lactis, farciminis, helveticus, jensenii, johnsonii, lactis ssp. lactis, lactis ssp. lactis biovar diacetyllactis, leichmanii, mucosae, paracasei, paracasei ssp. paracasei, pentosus, plantarum, reuteri, rhamnosus, sakei, sakei subsp. sakei, salivarius, sanfrancisco and xylosus)

Use in food or feed: Lactobacilli are used in the food for acidification, and/or enhancement of flavor, texture and nutrition. They can also serve as starters or complementary cultures for several varieties of cheese, fermented plant foods, fermented meats, in wine and beer production, sourdough bread and silage, and as probiotics in feeds for livestock.

Epidemiological studies: n/a

Case reports: n/a

Literature arguing against a possible sensitization by the agent: there is ample evidence indicating that lactobacilli (or at least some strains of lactobacilli) alleviate allergic symptoms. Such properties were observed for *L. brevis* in the atopic dermatitis mouse model, where oral administration of the bacteria inhibited IgE-production, and skewed the immune response towards Th1 dominance [76]. *L. brevis*-fermented Kimchi also strongly alleviated symptoms of pruritus, anaphylaxis and inflammation in a mouse model [76,77].

L. casei was shown to attenuate lung inflammation and Th2 cytokine profiles in Der p2 sensitized mice, hinting at immunomodulatory properties useful to prevent respiratory allergies [78].

One study *in-vitro* in atopic dermatitis patients [79] and one study in mice [80] supported similar conclusions about *L. fermentum*.

In a very recent study [81], Van Overtvelt *et al* showed that *L. helveticus* reduced airways hyperresponsiveness, bronchial inflammation and proliferation of specific T cells in cervical lymph nodes in the murine asthma model and characterized this strain as a probiotic acting as a Th1/possibly Treg adjuvant that potentiates tolerance induction via the sublingual route.

In the OVA-induced allergy mouse model, Nonaka *et al* [82] showed that a *L. pentosus* strain induced IL-12 and IL-10 in-vitro and modulated the Th1/Th2 balance toward a Th1-dominant state. In-vivo, serum IgE levels were diminished and active cutaneous anaphylaxis reaction were suppressed. Splenic IL-10 production from splenocytes of OVA-immunized mice was upregulated by oral administration of *L. pentosus*.

Several clinical trials have confirmed the immunomodulatory properties of *L. paracasei*. *L. paracasei* alleviated symptoms of pollinosis [83], atopic dermatitis [84], allergic rhinitis [85,86]. Investigations of the immunological mechanisms behind these effects in the mouse model pointed at induction of IL-12 production [87]; suppression of IL-4 production [88] and APC maturation [89].

L. acidophilus prevented the pollen-induced infiltration of eosinophils into the nasal mucosa, and indicated a trend for reduced nasal symptoms in children [90] and similarly improved Japanese cedar pollinosis in adults [91]. In a small clinical trial, 14 patients with various allergic diseases showed a decrease in their circulating CD34+ precursor cells after oral treatment with a mix of *L. acidophilus* and two other bacteria [92]. Elevated CD34+ precursor numbers is a feature of systemic allergic inflammation. Two mouse studies showed similar effects [93,94] on house dust mite and OVA sensitization respectively. However; it is worth noting that at least one study failed to confirm the role of *L. acidophilus* in allergy prevention [95].

L. plantarum has been shown to reduce the severity of type 1 allergic reactions both in humans [96] and mice [97,98].

In 2003, a double-blind, placebo-controlled, crossover study in children showed that a combination of *L. rhamnosus* and *L. reuteri* was reduced symptoms of atopic dermatitis, especially in patients with increased IgE levels [99]. Supplementation of *L. reuteri* during pregnancy was associated with low levels of TGF-beta2 and slightly increased levels of IL-10 in colostrum [100]. Studies in BALB/c mice argued for a role for non antigen-specific CD4(+)CD25(+)Foxp3(+) regulatory T cells in attenuating the allergic airway response following oral treatment with *L. reuteri* [101]. Oral treatment with the live bacteria greatly decreased allergen-induced airway hyperresponsiveness, but a similar effect was not observed with the killed organism [102].

Lactococcus lactis

Use in food or feed: *L. lactis* is used in the early stages for the production of many cheeses including Brie, Camembert cheese, Cheddar, Colby, Gruyère, Parmesan, and Roquefort. Other uses include the production of pickled vegetables, alcoholic beverages, and other fermented food-stuffs. It can also be used as a probiotic in feeds for livestock.

Epidemiological studies: n/a

Case reports: n/a

Literature arguing against a possible sensitization by the agent: two recent studies in mice indicate that treatment with *L. lactis* together with an allergen (BLG) decreased IL-4 production and enhanced IFN-gamma production by BLG-reactivated splenocytes, suggesting a switch from Th2- to Th1-immune response [103,104]. Symptoms after intranasal challenge were locally reduced, as evidenced by decreased release of IL-4 in bronchoalveolar lavage fluids.

Leuconostoc pseudomesenteroides

Use in food or feed: *L. mesenteroides* is a bacterium associated with the sauerkraut and pickle fermentations.

Epidemiological studies: n/a

Case reports: n/a

Literature arguing against a possible sensitization by the agent: in an *in-vitro* study in mouse splenocytes, a strain of *L. mesenteroides* proved to be a useful Th1 stimulating agent, especially by upregulating IFN-gamma production [105].

Pediococcus pentosaceus

Use in food or feed: *P. pentosaceus* is used as an acid producing starter culture in sausage fermentations, cucumber and green bean fermentations, soya milk fermentations, and silage.

Epidemiological studies: n/a

Case reports: n/a

Animal studies: Duchaine *et al* [106] showed that *P. pentosaceus* has a pro-inflammatory effect in mice similar to that of *S. rectivirgula*, the most common etiological agent of farmer's lung. This was in contrast of a most recent publication by Masuda *et al* [107], where they showed that the Sn26 strain of this bacteria increased the production of Th1 cytokines in Peyer's patches of allergic diarrheic mice.

Literature arguing against a possible sensitization by the agent: n/a

Penicillium candidum

Use in food or feed: *P. candidum* is used in cheese-processing.

Epidemiological studies: In a study from 1994, 16 cheese-factory workers out of 24 had airway symptoms, five had asthma requiring treatment [108]. Two-thirds of the symptomatic cheese-workers had precipitating antibodies; compared to only half in the non-symptomatic group.

Case reports: n/a

Literature arguing against a possible sensitization by the agent: n/a

Penicillium nalgiovensis

Use in food or feed: *P. nalgiovensis* is the most frequently used starter for cured and fermented meat.

Epidemiological studies: a study of 59 workers from a sausage factory showed that symptoms of HP such as sneezing, cough, dyspnoea, nasal obstruction, headache, and discomfort were significantly more frequent in workers exposed to *P. nalgiovensis* [109].

Case reports: a 45-year-old male pork butcher was reported to experience cough, tightness of the chest, and sibilant dyspnea for 2 years [110]. Episodes were related to the handling of sausages and inhalation of the dust coming from the sausage casings at his work in the butcher's area of a supermarket. The patient had IgE to *P. nalgiovensis*.

Literature arguing against a possible sensitization by the agent: n/a

Penicillium roqueforti

Use in food or feed: *P. roqueforti* is typically used in the production of blue cheeses.

Epidemiological studies: n/a

Case reports: Campbell *et al* [111] described the case of a cheese factory worker with symptoms of cough, dyspnea, and malaise, and findings of bibasilar crackles, reduced lung volumes, hypoxemia, and bilateral infiltrates on chest roentgenogram. All symptoms resolved after she left the workplace. Bronchoalveolar lavage revealed a high percentage of lymphocytes. Antibodies to *P. roqueforti* were detected in serum and lavage fluid.

Literature arguing against a possible sensitization by the agent: n/a

Saccharomyces cerevisiae

Use in food or feed: *S. cerevisiae* is a top-fermenting yeast, and has been used for brewing and baking for centuries. It is also widely used as a probiotic for livestock.

Epidemiological studies: a study of 449 subjects, including 226 atopic dermatitis (AD) patients, 50 patients with allergic rhinitis and/or asthma, and 173 nonatopic controls found that a positive SPT reaction (> or = ++) was seen in 94% of patients with severe AD, in 76% with moderate AD, and in 25% with mild AD or no history of AD. Patients with rhinitis and/or asthma and nonatopic controls displayed a positive reaction in only 8 and 2% of cases, respectively [112].

Case studies: In 1996, a 48-year old baker was reported to have hydrorrhea, sneezing, nasal obstruction, wheezing, spasmodic cough, and dyspnea upon exposure to the dried form of the

yeast. The patient began to use conventional wet yeast and carried on normal work activity without symptoms [113]. An interesting case was described in 2005. The patient experienced generalized urticaria and asthma after eating pizza and bread, but only fresh from the oven, and had IgE to *S. cerevisiae* [114]. If bread, pizza and cakes were eaten more than one hour after preparation, no symptom would occur at all. Why the allergen was active only in ready-baked foods remains unexplained.

Literature arguing against a possible sensitization by the agent: n/a

4.3. Enzymes allowed in foods & feeds

This section describes the results of the systematic literature search that was conducted on Pubmed, together with the extra information that we found by backtracking references from important reviews through Web of Knowledge. For each enzyme from our list, we listed all the relevant information we could find in the following order:

enzyme

EC number, application (food and/or feed)

use in food or feed (examples of use of the enzyme in food or feed, non-exhaustive)

production organism

epidemiological studies

case reports

animal studies

in-silico work

allergen of a similar class outside of the food and feed industry

21 enzymes were found to have relevant information, for a total of 75 relevant articles. Only enzymes for which relevant information could be found are listed here.

3-Phytase, 6-Phytase, Phytase

EC: 3.1.3.x – Food and Feed

Use in food or feed: increase the bioavailability of phosphorus in cereals and soy for monogastric animals.

Production organism: *Trichoderma reesei*, *Aspergillus orizae*, *Aspergillus niger*, *Schizosaccharomyces pombe*, *Trichoderma longibrachiatum*.

Epidemiological studies: Doekes *et al* [115] report a prevalence of 55% of respiratory symptoms among 11 exposed workers in a factory producing premix for feed additives. 73% of those 11 workers had measurable IgE against phytase. Zober *et al* [116] performed a cross-sectional study on 49 workers from an R&D facility with potential contact with phytase and found symptoms of conjunctivitis, rhinitis, or bronchitis in 65% of those employees. Baur *et al* [117] examined 53 employees with occupational exposure to phytase either during large-scale enzyme production (powdered form), enzyme laboratory analysis, or animal husbandry. Symptoms were reported in 72% of the subjects, including dyspnoea (23% of the subjects), rhinitis (66%) and conjunctivitis (30%). 28% of the subjects exhibited IgE-mediated

sensitization, and 47% had measurable IgG levels. Finally, Caballero *et al* [118] examined 86 workers from two animal feed factories and found that seven of those were sensitized to 3-phytase or 6-phytase. Three of those workers had asthma symptoms, two had asthma and rhinoconjunctivitis, one had asthma and rhinitis, and one had rhinitis only.

Case reports: O'Connor *et al* [119] describe the case of a 43-year-old man working in the field of animal feed manufacturing, presenting symptoms of wheezing and coughing, and displaying high levels of IgE against phytase. Van Heemst *et al* [43] also report a 43-year-old man working in the cattle feed industry, with symptoms of hypersensitivity pneumonitis (coughing, shortness of breath, fever) and high levels of serum IgG against phytase.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Amylase

EC: 3.2.1.1 (alpha-amylase), 3.2.1.2 (beta-amylase) – Food and feed.

Use in food or feed: accelerate the breakdown of starch, for example to enhance yeast performance in the baking industry.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Microbacterium imperiale*, *Trichoderma reesei*, *Trichoderma longibrachiatum* (alpha-amylase); barley, soybean (beta-amylase)

Epidemiological studies: there is a large number of studies of amylase-linked allergic reactions, especially in the bakery industry. In 1996, a cross-sectional study of 178 bakery workers in the Netherlands found a positive association between positive skin prick tests to alpha-amylase and work-related respiratory symptoms [120], establishing conclusively the strong and positive relationship between alpha-amylase allergen exposure levels in bakeries and specific sensitization in bakery workers. This finding was confirmed 3 years later in a study looking at 495 flour samples from British bakeries [121]. In 2004, Brisman *et al* [122] took a closer look at a cohort of 300 bakers and found 36 new cases of chest symptoms, 86 of eyes/nose symptoms, and 24 of positive SPT to a-amylase. They showed exposure-response relations for chest and eyes/nose symptoms and for sensitization, and an increased prevalence for chest symptoms in the most exposed workers. Similar relations were confirmed one year later in 227 bakery workers in Belgium in 2005 [123].

A large 2009 study of 517 supermarket bakery workers in South Africa revealed that only 4% of the workers had a sensitization to fungal amylase [124], underlining the effect of work environment regulations. By comparison, IgE antibodies to fungal amylase are found in 1% of US blood donors [125]. Similarly, it has been observed that alpha-amylase from *Bacillus amyloliquefaciens* did not cause sensitization in a study involving 84 animal feed workers [126] whereas other fungal enzymes were found to be sensitizers in the same settings. For

more studies linking amylases to occupational allergies see [127,128,129,130,131,132,133,134].

Case reports: since the link between amylases and allergy is well-documented thru numerous epidemiological studies, case studies worth mentioning fall a bit outside of the classical baker's asthma. Interesting case reports on amylase include a double sensitization to lysozyme and amylase in a baker with rhinoconjunctivitis and asthma [135], a sensitization occurring from infrequent contact with the enzyme in a lab technician showing symptoms localized on her hands without nasal or respiratory effects [136], and one case of occupational allergic contact urticaria from fungal but not bacterial alpha-amylase [137]. Two cases of IgE-mediated rhinitis have also been reported in hospital and pharmaceutical workers exposed to biodiastase, a prescription drug based on alpha-amylase [138].

Animal studies: it has been shown that amylase from bread crust and rolls crust kept between 0.1 and 20% of the antibody-binding capacity of amylase from dough [139].

In-silico work: Warren *et al* [140] have developed a dynamic population-based model for the development of work-related respiratory health effects among bakery workers. The model predicts that non-atopic/non-sensitised workers had probabilities of developing moderate symptoms and progression to severe symptoms of respectively 0.4% (95% CI 0.3 to 0.5%) and 1.1% (95% CI 0.6 to 1.9%) per mg/m³/year of flour dust. These probabilities were twice as high in atopic workers. They also predict that 36% (95% CI 26 to 46%) of workers with severe symptoms are sensitised to wheat and 22% (95% CI 12 to 37%) to alpha-amylase.

Allergens of a similar class outside of food and feed area: alpha-amylase from porcine pancreatic extract [141] and mite amylase [142] are both respiratory sensitizers.

Asparaginase

EC: 3.5.1.1 - Food

Use in food or feed: converts asparagine into aspartic acid. This prevents the formation of carcinogenic acrylamide during high temperature processes such as cookie manufacturing.

Production organism: *Aspergilly niger*, *Aspergillus oryzae*.

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: Asparaginase can also be used as a cancer chemotherapy agent. Lee *et al* have reviewed allergy cases of this application and found that reactions have been reported to occur in up to 35% of treated patients, although serious anaphylactic reactions occur in less than 10% of patients [143].

Beta-glucanase

EC: 3.2.1.6 – Food and feed

Use in food or feed: Beta-Glucanase reduces intestinal viscosity when added to animal feeds and increases the activity of other enzymes—such as amylase, lipase, and trypsin—leading to improved weight gain and nutrition.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus amyloliquefaciens* or *subtilis*, *Cellulosimicrobium cellulans*, *Disporotrichum dimorphosporum*, *Humicola insolens*, *Penicillium funiculosum*, *Talaromyces emersonii*, *Trichoderma harzianum*, *Trichoderma reesei*, *Trichoderma longibrachiatum*.

Epidemiological studies: n/a

Case reports: O'Connor *et al* [119] describe the case of a 43-year-old man working in the field of animal feed manufacturing, presenting symptoms of wheezing and coughing, and displaying high levels of IgE against beta-glucanase.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: Palomares *et al* [144] report a case of allergy to Ole e 9, an olive pollen beta-glucanase in a researcher. Ole e 9 is a well-known seasonal respiratory disease in Mediterranean countries.

Catalase

EC: 1.11.1.6 – Food and feed

Use in food or feed: Catalase is used in the food industry for removing hydrogen peroxide from milk prior to cheese production. Another use is in food wrappers where it prevents food from oxidizing.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*.

Epidemiological studies: el-Said *et al* [145] reported high levels of IgE to catalase in a study on 25 bakers.

Case reports: n/a

Immunological studies in humans: Ward *et al* [146] investigated sera from asthma patients, and showed that there was a high level of cross-reactivity among fungal catalases. The authors indicate that this cross-reactivity might be due to the high level of conservation of the catalase gene among fungi as shown by Bowyer *et al* [147].

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Cellulase

EC: 3.2.1.4 – Food and feed

Use in food or feed: Cellulases are used in many technical processes to make soluble the cellulose that is present in plant-based raw materials. They are widely use in the production of beverages and help increase the digestability of plant-based products.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*, *Humicola insolens*, *Penicillium funiculosum*, *Talaromyces emersonii*, *Streptomyces lividans*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, *Trichoderma viride*.

Epidemiological studies: Elms *et al* have assessed the prevalence of sensitization to fungal enzymes in 135 bakery workers and found that 8% of the workers' sera had IgE reacting to cellulase [148].

Case reports: 7 cases of occupational allergy to cellulase have been found in the litterature [149,150,151,152,153,154]. All 7 cases have in common symptoms of rhinitis and shortness of breath/bronchoconstriction.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Chitinase

EC: 3.2.1.14 - Food

Use in food: antifungal activity

Production organism: *Streptomyces violaceoruber*

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class: chitinase from maize and grape [155], as well as of chestnut, avocado and banana [156] have been linked to food allergies.

Other references to chitinase in relation to allergy in the literature: Endochitinase has been characterized as having a role in asthma [157] and in Th2 responses in general [158] and is therefore the focus of a consequent number of articles related to allergy research. It is worth noting however that chitinase is not a sensitizer in this case, but merely an endogenous enzyme implicated in the immune processes leading to allergic responses.

Esterase

EC: 3.1.1.1 - Food

Use in food: transesterification of triglycerides with free fatty acids in the food industry.

Production organism: *Rhizomucor miehei*.

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: the major latex allergen Hev b 13 is an esterase [159]. An important allergen from *Carica papaya* pollen also has an esterase activity [160].

Glucoamylase

EC: 3.2.1.3 - Food

Use in food: finds use in bread making and to break down complex sugars such as starch (found in flour) into simple sugars.

Production organism: *Aspergillus niger*, *Rhizopus niveus*, *Rhizopus oryzae*, *Trichoderma reesei* or *longibrachiatum*.

Epidemiological studies: Sander *et al* [161] examined 171 bakers exhibiting symptoms of asthma, rhinitis and conjunctivitis. 8% of them were found to have specific IgE to glucoamylase.

Case reports: Quirce *et al* [162] have described four cases of patients (three bakers, one enzyme processing plant worker) presenting symptoms such as cough, shortness of breath and wheezing. All four patients had IgE against glucoamylase.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Other routes of exposure: Kanerva *et al* [163] described the case of a chemical enzyme factory process operator presenting symptoms of itching and dermatitis.

Glucose oxidase

EC: 1.1.3.4 – Food and feed

Use in food or feed: Glucose oxidase is used for the removal of glucose or oxygen from the foodstuff in order to enhance their stability during storage.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium chrysogenum*.

Epidemiological studies: n/a

Case reports: Simon *et al* [164] reported the case of a 29-year-old man with granulomatous lung disease, employed in the extraction and purification of glucose oxidase from *Aspergillus niger* two years before admission. The link between the symptoms and the enzyme has not been formally proved in this study.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Hemicellulase

EC: No EC number provided by AMFEP- Food and feed

Use in food or feed: used in bakery for the enhancement of dough qualities (mechanical handling, stability) and for product optimisation (volume, consistency, storage life), as well as in the production of fruit juice and other beverages, in the production of spirits and in the alcohol industry, in wine production or as an additive to animal feed (to increase digestibility).

Production organism: *Aspergillus niger*, *Bacillus amyloliquefaciens* or *subtilis*, *Trichoderma reesei* or *longibrachiatum*.

Epidemiological studies: In a cross-sectional study, Elms *et al* [148] analyzed the serum of 135 bakery workers. 8% of those workers had IgE against hemicellulase, and there was a significant correlation between specific IgE and nasal symptoms.

Case reports: Quirce *et al* [162] have described four cases of patients (three bakers, one enzyme processing plant worker) presenting symptoms such as cough, shortness of breath and wheezing. Two of those four patients had a positive skin-prick test to hemicellulase.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Invertase or Fructofuranosidase (beta)

EC: 3.2.1.26 – Food and feed

Use in food or feed: hydrolysis of sucrose. The resulting mixture of fructose and glucose is called inverted sugar syrup and is a constituent of the liquid sugar found in chocolate bars.

Production organism: *Saccharomyces cerevisiae*.

Epidemiological studies: Horner *et al* [165] examined 20 patients showing symptoms of respiratory allergies and skin test reactivity to at least 2 fungal allergens. 80% of those patients showed RAST reactivity to invertase.

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class in a different species/different route of exposure: Westphal *et al* reported that 17% of 78 patients presenting food allergy to tomato had IgE against a tomato invertase.

Lactase or Galactosidase (beta)

EC: 3.2.1.23 - Food

Use in food or feed: Lactase is used commercially to prepare lactose-free products, particularly milk. It is also used in preparation of ice cream, to make a creamier and sweeter-tasting product.

Production organism: *Aspergillus oryzae*, *Kluyveromyces lactis*, *Bacillus circulans*.

Epidemiological studies: in 1997, Muir *et al* [166] looked at 207 volunteers from a lactase packaging plant and observed that 30% had positive results to skin prick test with lactase. The lactase skin test results correlated with nasal and eye symptoms, but not with symptoms suggestive of asthma. In 1999, Bernstein *et al* [167] found similar results in a study involving 94 pharmaceutical workers occupationally exposed to lactase. 29% of the workers were skin prick positive, and those were 9 times more likely to have respiratory symptoms than workers with negative skin-pricks.

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Other routes of exposure: Binkley *et al* [168] report the case of a 35 year-old man with a burning sensation in his mouth and throat and difficulty swallowing after consumption of lactase pills of lactase supplemented milk. The patient was positive to lactase skin prick test.

Lysozyme

EC: 3.2.1.17- Food

Use in food: lysozyme is used as a food preservative due to its antibacterial properties.

Production organism: chicken egg

Epidemiological studies: in a double blind, placebo-controlled food challenge, concentrated lysozyme was allergenic in a skin prick test, but no patient reacted adversely in the provocation test to fined wine [169]. The authors concluded that wines treated with fining agents at commercial concentrations did not present a risk to allergic individuals when filtered. Similar conclusions were drawn about the contents of lysozyme in Grana Padano cheese [170]. However; it has been suggested that lysozyme may present a harmful adjuvant in wine processing for consumers allergic to hen's egg [171].

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Pectin methylesterase or Pectinesterase

EC: 3.1.1.11 – Food and feed

Use in food or feed: increases fruit juice yield and clarifies juices through the elimination of lees; is used in the production of concentrates from fruits and vegetables, as well as colouring extracts and food colour from plant-based raw materials.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *Penicillium funiculosum*, *Rhizopus oryzae*, *Trichoderma reesei* or *longibrachiatum*.

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: Barderas *et al* [172] report that while examining 11 patients allergic to russian Thistle (*Salsola kali*) pollen, they found that all of them had IgE against Sal k 1, a protein from the pectin methylesterase family.

Pectinase

EC: 3.2.1.15 – Food and feed

Use in food or feed: break down pectin, a polysaccharide substrate that is found in the cell walls of plants. Used to facilitate the extraction of juice from fruits.

Production organism: *Aspergillus niger*, *Rhizopus oryzae*, *Trichoderma reesei* or *longibrachiatum*.

Epidemiological studies: Belleri *et al* [173] examined 13 workers from a pectinase production factory and showed that 61% of them had IgE to pectinase. 38% of those 13 workers had both respiratory symptoms and detectable IgE against pectinase.

Case reports: Sen *et al* [174] reported 3 cases of asthma and conjunctivitis among workers from a fruit salad processing plant. All three had IgE against pectinase. It is interesting to note that in this factory, pectinase was used in a liquid form.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: Ibarrola *et al* [175] examined 26 patients with allergy to *Platanus acerifolia* pollen. 81% of the patients had rhinoconjunctivitis, 57% had asthma, 48% had rhinitis, 5% had urticaria. 81% of the patients had IgE against Pla a 2, a pectinase.

Peroxidase

EC: 1.11.1.7 - Food

Use in food: increases food stability

Production organism: *Aspergillus oryzae*, Soybean hulls

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class: a peroxidase from wheat has been reported to be an allergen, with sera from 6 out of 10 wheat-allergic patients reacting to the purified dot-blotted allergen [176].

Phospholipase A

EC: 3.1.1.4 - Food

Use in food: bread making, egg yolk industry (emulsification for different applications) and refinement of vegetable oils.

Production organism/source: *Aspergillus niger*, *Streptomyces vialoceanus*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, pig pancreas, ox pancreas.

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class: Phospholipases are very common allergens of venom from bees and other arthropods [177].

Transglutaminase

EC: 2.3.2.13 – Food and feed

Use in food or feed: is used mainly to improve the physical quality of products such as firmness, elasticity and texture. To a lesser extent, it can also modify taste.

Production organism: *Streptovercillium mobaraense*

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Assessment of safety: Pedersen et al [178] followed the 2001 FAO/WHO decision tree to assess the safety of transglutaminase and found no concern with regard to its allergenic potential.

Xylanase

EC: 3.2.1.8 – Food and feed

Use in food or feed: increases the digestibility of silage, improve the dough's workability and absorption of water in bakery.

Production organism: *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus amyloliquefaciens* or *subtilis*, *Bacillus licheniformis*, *Disporotrichum dimorphosporum*, *Humicola insolens*, *Penicillium funiculosum*, *Talaromyces emersonii*, *Trichoderma reesei*, *Trichoderma viride*.

Epidemiological studies: In a cross-sectional study, Elms *et al* [148] analyzed the serum of 135 bakery workers. 6% of those workers had IgE against xylanase, and there was a significant correlation between specific IgE and nasal symptoms. In a retrospective study, Sander *et al* [161] examined 171 bakers exhibiting symptoms of asthma, rhinitis and conjunctivitis. 13% of them were found to have specific IgE to glucoamylase. Vanhanen *et al* [179] conducted a cross sectional study on 365 workers in four bakeries, one flour mill, and one crispbread factory. 16% of the workers presented respiratory symptoms, and 14% had a positive skin prick test to a mix of enzymes including xylanase. In a similar study in the animal feed industry, the same group found that 7% of 140 exposed workers were sensitised to enzymes to a mix of enzymes including xylanase.

Case reports: Baur *et al* [180] report the case of a bakery worker with rhinoconjunctivitis, cough, and shortness of breath. The patient reacted to an inhalative challenge with $\approx 0.5 \mu\text{g}$ of xylanase. Merget *et al* [154] describe the case of a baker with occupational asthma (nasal congestion, sneezing, running nose, watering of the eyes and shortness of breath). The patient showed significant levels of IgE against xylanase.

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: n/a

Proteases

EC: 3.4.2x.xx – Food and feed

Use in food and feed: proteases are used for a wide range of applications such as milk coagulation, fish processing, or improvement of the digestibility of animal and vegetal proteins.

Production organism/source: *Actinida chinensis*, *Ananas bracteatus*, *Ananas comosus*, *Aspergillus melleus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *Bacillus amyloliquefaciens* or *subtilis*, *Bacillus clausii*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Carica papaya*, *Cryphonectria* or *Endothia parasitica*, *Ficus glabrata*, *Fusarium venenatum*, *Geobacillus caldoproteolyticus*, *Kluyveromyces lactis*, *Rhizomucor miehei*, *Rhizopus niveus*, *Trichoderma reesei* or *longibrachiatum*, lamb stomach, goat stomach, kid stomach, ox stomach, calf stomach, pig pancreas.

Epidemiological studies: n/a

Case reports: n/a

Animal studies: n/a

In-silico work: n/a

Allergens of a similar class outside of food and feed area: Proteases are a very well known class of allergenic enzymes, especially but not exclusively in the context of detergent manufacturing [36]. In addition to their intrinsic allergenicity, proteases have potential effects on several processes involved in allergic diseases: epithelial integrity and permeability, mast cell degranulation, cytokine release from the respiratory epithelium [181,182]. Proteases have been shown to interact with receptors from the lining of the respiratory tract, leading to the activation of immunological pathways that can potentially trigger allergic reactions, for example via stimulating epithelial cells to release metalloproteinase 9 and open up tight junctions promoting allergen penetration into the submucosa [182]. Proteases from pollen [183], house dust mites [184], cockroaches [185] and fungi [186] have been shown to have these effects.

4.4. Existing legislation and regulation

We have conducted a survey of the existing legislation and regulations regarding enzymes and microorganisms and the basis for occupational exposure limits (OELs) and labelling. The integrale version of this survey can be found in appendix 3. It appears from this survey that OELs have been set for enzymes (subtilisins) in the UK and the US, but that these limits are bypassed by the limits set “in-house” by the detergent industry. It is also expected that the European regulations will in the future set some derived no effect level (DNEL) or derived minimal effect level (DMEL) for macromolecular compounds including enzymes.

The appendix also includes a list of proteins as airway allergens together with the corresponding labelling and OEL, whenever they exist.

5. Conclusions

5.1. Models for predicting respiratory sensitization

It appears from our research that there is currently no established model to predict the allergenicity of a molecule. Although *in-silico* models can be useful to predict cross-reactivity between allergens, they only take into account the structure of the allergen, which is only one of the many factors implicated in sensitization, and in our opinion only a minor one. *In-silico* models fail to take into account the context in which the allergen is presented to the immune system.

In-vitro models are currently far from being used for predictive studies. Although many cell lines are currently used in the lab, research is still trying to understand the role and mechanisms of the cells in the development of allergies, rather than using them to test the allergenicity of various molecules. Although co-culture three-dimensional models might someday achieve that purpose, they remain very far from re-creating the complexity of the immune response in a living organism.

Among *in-vivo* inhalation animal models, the mouse is currently the best. However, it suffers from several shortcomings that prevent it from being a reliable predictive model: most models require systemic sensitization by injection, followed by multiple respiratory challenges, which hardly resembles the exposure conditions in humans. Additionally, most mouse models are strong “atopic-like” responders that can be sensitized to molecules that are not allergenic in humans.

5.2. Microorganisms

In general, exposure to bacterial species leads to a Th1-type response in humans, increasing macrophages efficiency and leading to the production of opsonising antibodies, two systems very useful to deal with that type of pathogens. In contrast, Th2 responses to bacterial infections are rare, and can sometimes be indicative of a poor prognosis, such as in the case of leprosy.

As an illustration of the apparent lack of role of bacteria in allergic diseases, we have been unable to find an example of regulations such as OEL to work with such organisms. The most recent revision of the *Council Directive 93/88/EEC of 12 October 1993, Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC)* provides a list of microorganisms together with their classification. There are no bacteria classified as

having *possible allergic effects*. Thus, it is not considered a general feature of bacteria to express sensitising properties.

Although one study reporting type-1 reactions to *B. subtilis* extracts was found in our survey, the overwhelming majority of reports were arguing for a beneficial role of bacteria to allergy symptoms, through the induction of Th1 and/or Treg responses. This observation is of course partially biased by the fact that lactic acid bacteria were the most studied microorganisms in our list, and these are actually used for their immunomodulatory properties. It is also worth noting that these properties are strain-dependant: only a few strains of a given species have an immunomodulatory effect, while the others have usually no beneficial effect on allergy symptoms. In addition, one recent study introduced the idea that Th1-inducing bacteria only might not be sufficient for inducing tolerance, but that Treg-induction might also be required [81].

It seems that the best-documented allergic risk linked to occupational exposure to bacteria is hypersensitivity pneumonitis. From our survey, it seems that HP has been linked to exposure to *B. pumilis* (machine operator's lung), *B. subtilis* (wood dust) and *A. globiformis* (agricultural workers). HP remains, however, a very rare disease, and is associated with very specific conditions and high level of exposure. None of the cases described resulted from an intentional use of the bacteria.

The case of fungi is quite different. Strong evidence of a link between asthma severity and fungal exposure has been previously established. Sensitization to fungal species has been shown to be a significant risk factor for severe asthma [187]. Most likely, fungal sensitization can have a causative role in severe asthma [188].

Furthermore, sensitization and exposure to fungi have been linked to hospital admissions and emergency department visits for asthma, life-threatening asthma, admission to ICU, and asthma-related mortality. The role of fungi in severe asthma hospitalizations and mortality has been reviewed in [189].

Indoor fungal exposure is particularly relevant to this report. Verhoeff *et al* reviewed 9 studies on indoor fungi exposure and hypersensitivity. The majority of those studies argued for a link between domestic fungal levels and allergic symptoms [190]. However, it is worth noting that at least one review disputes the studies linking indoor moulds exposure to upper airway allergy (rhinitis), while agreeing with its role in asthma [191].

It is interesting to see that, as opposed to bacteria; there are regulations regarding the acceptable levels of exposure to several fungi (moulds). The above-mentioned European classification has been applied to 8 (groups of) fungal species: *Aspergillus fumigatus*, *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans* var. *neofonnans* (*Filobasidiella neofonnans* var. *neofonnans*), *Cryptococcus neoformans* var. *gattii* (*Filobasidiella bacillispora*), *Epidermophyton floccosum*, *Microsporum* spp., *Penicillium marneffeii*.

Our survey of the literature unveiled 8 species of fungi associated with type 1 allergies, including several studies demonstrating the link between occupational exposure to fungi and hypersensitivity. Cases of IgE-mediated hypersensitivity as well as hypersensitivity pneumonitis are well documented and involve several distinct genera.

In general it seems that fungi can be sensitizers, although allergic symptoms are associated with high levels of exposure compared to other respiratory sensitizers, such as pollen. In contrast to the situation with bacteria, some of the cases that we have presented result from an intentional use of the microorganism. In particular, the cases of *P. roqueforti* (cheese-making), *S. cerevisiae* and *A. oryzae* are very relevant for this report, as they are directly linked to food production processes.

In addition to compiling scientific literature on the topic, we also contacted one of the leading companies in the field of industrial cultures of microorganisms, Chr. Hansen A/S. The company claims no cases of allergy related to microorganisms among employees. The only incident involving respiratory symptoms (likely be caused by a toxic/irritative mechanism) occurred when a customer in Brazil sprayed the cultures in an indoor environment. This was a highly unusual process, and very different from the intended use of the product. The lack of documented allergic reactions to microorganisms can of course be tied to the comprehensive preventive measures applied throughout the production process. Few workers are directly exposed to inhalable products, and those who do obey strict rules, including wearing personal protection equipment. Although very commendable, it is possible that those measures partly hide the sensitizing potential of these microorganisms.

5.3. Enzymes

Sensitizing properties of enzymes have been a cause of concern for several decades in food and feed industry, albeit at lower levels than in the bakery or detergent industry [192]. Allergies to *B. subtilis* proteases, for example, have been described since the late 1960s [193]. The most reported cause of respiratory sensitization by enzymes in the food and feed industry is perhaps the case of alpha-amylase and baker's asthma, but other enzymes such as proteases, cellulase and glucose oxydase are also a cause of concern. To compound the problem, industrial enzyme grades are often poor and contain other enzymes from the same production organism, leading to unexpected sensitization and cross-reactions [179]. Studies conducted in enzyme-producing plants have confirmed that enzymes can induce sensitization and allergy [194], regardless of their enzymatic activities, or production organism.

Although not related to food and feed, and therefore out of the scope of this report, the detergent industry also has an extensive experience with enzymes. While respiratory allergies in consumers proved to be a problem in the early years of enzyme addition to detergents (1970s), granulated formulations have reduced type-1 reactions in the general population to

virtually nothing [195]. The problem is now limited to manufacturing processes, and stringent rules in the modern enzyme- and detergent-producing field have contributed to greatly diminish sensitization in workers.

Like for microorganisms, hypersensitivity pneumonitis to enzymes has been described, for example to phytase [43]. Cases remain however extremely rare, and seem associated with very high levels of exposure.

Out of 71 enzymes listed as food or feed additive by the AMFEP, our survey found 18 that were related to allergies. All but one of these were linked to respiratory sensitization. Although our survey seems to indicate that the general trend is towards a decrease in exposure levels and sensitization to enzyme in the industry, it is worth noting that studies in the field suffer from inherent selection biases and therefore probably underestimate the true prevalence of sensitization to enzymes.

As we did previously for microorganisms, we completed our survey of the literature with a direct contact with a leading producer of enzyme, Novozymes. According to the company, less than 10 cases of symptomatic workers are identified every year, and the symptoms/sensitivity ratio has been decreasing due to reduced exposure in the recent years, pointing at a dose-response effect. Interestingly, these numbers seem higher than those observed in the case of microbial cultures, although it is difficult to make a comparison between two different production processes taking place in two different companies. No cases have been reported among customers since 1970.

Novozymes A/S acknowledges the sensitising role of enzymes and relies on stringent personal protection measures to minimize exposure of workers. The use of granulate formulation has led to a steep decrease in exposure levels, with differences between formulations (more effective for detergent than bakery uses). The company research has observed no differences in relative allergenicity between enzymes, which seems to indicate that the physical properties of enzymes (formulation, molecular weight...) might be more important than their function or amino-acid sequences. The role of the production organism in allergenicity is currently being investigated.

6. Final Remarks

When considering the findings on inhalation allergy risks presented in this report, a number of observations stand out:

- * The enzymatic activity in terms of substrate specificity etc, does not seem to explain whether an enzyme has been incriminated as an inhalation allergen or not.
- * Neither does the production organism seem to decide whether or not an enzyme has been reported as an inhalation allergen.
- * While it is possible to devise sensitization regimes in animals, in particular in mouse, neither these, nor *in-silico* or *in-vitro* systems seem to work well in predicting the allergenicity of enzymes in real life.

This would suggest that the inherent properties of an enzyme may not be decisive, as to whether it is going to create inhalation allergies.

Looking at where and when enzyme allergies have been reported, there seems to be a clear gradient from the producers of enzymes, over manufacturers that formulate their products with inclusion of enzymes, to end users that may be exposed to enzyme-containing products (Fig. 1). Also - and in spite of an increase in the worldwide use of enzymes - there seems to be fewer recorded cases of allergies and sensitizations compared to the situation in the 1960'ies and 1970'ies where much higher levels of airborne enzymes prevailed.

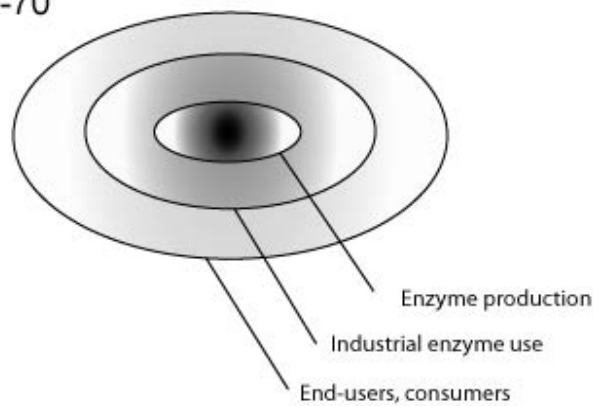
It seems reasonable to conclude that this reduction is caused by a reduced exposure, which is likely to be caused by different formulations of the raw enzymes which are now dispensed in liquid or granulated forms rather than the former use of powder of small particle sizes.

The exact regimens, i.e. temporal concentration profiles, that cause sensitization and may lead to inhalation allergies are not known, but in spite of the fact that in allergy development dose-relationships may not always be linear, it is likely that increasing doses of inhaled enzyme lead to increased risks. Also - estimated from the lack of reports of enzymes allergies in the general population - it seems that there are thresholds under which exposure does not cause sensitization and allergy. From this follows that setting demands on the formulation (distribution in non-inhalable forms) and designing of the processes in which enzyme-containing (food or feed) products are used, would be a way to reduce the sensitization and allergy risk. If these measures do not lead to consistently low airborne exposure throughout the life cycle of the product, it is necessary to label products and protect exposed persons by personal equipment such as masks etc.

It is not known whether sensitization always lead to clinical allergy, but based on industrial hygiene experiences and knowledge from other areas of allergy, it must be assumed that sensitization, i.e. the formation of IgE antibodies against an enzyme, would be a very strong risk factor for also developing symptoms upon continued exposure.

The different steps in developing enzyme allergy and the potential prevention measures are depicted in Fig. 2.

Situation 1960-70



Situation 2010

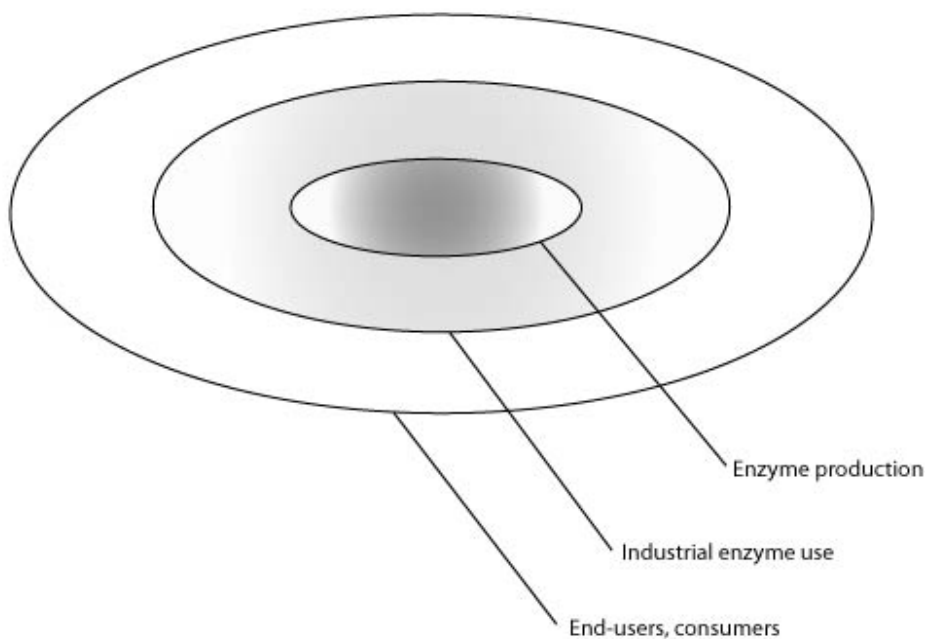


Figure 1: flow of enzymes in society, from the producer to the end-user. The shade of darks represent the number of prevalence of sensitization for each category of person exposed.

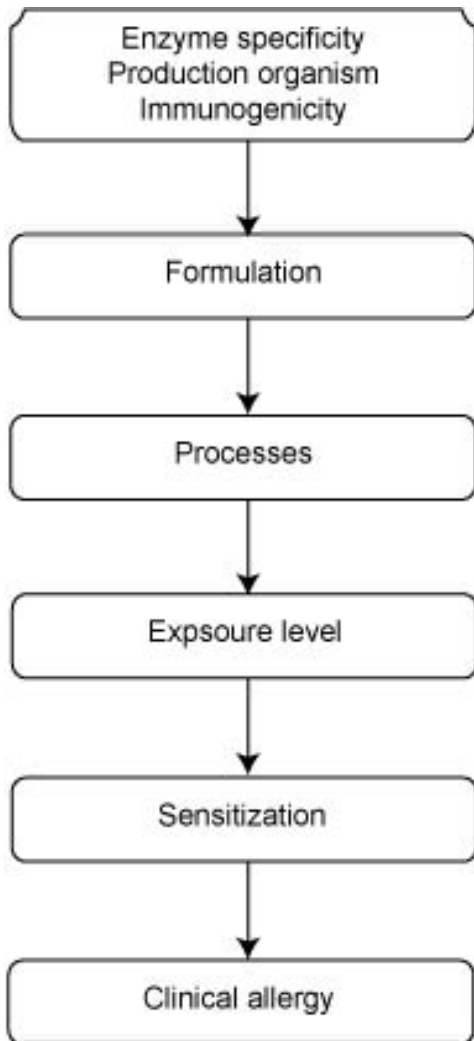


Figure 2: key steps in developing enzyme allergy.

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8. Appendices

Appendix 1. List of microorganisms included in literature search

Acetobacter aceti

Arthrobacter globiformis

Aspergillus niger

Aspergillus oryzae

Bacillus amyloliquefaciens

Bacillus cereus

Bacillus coagulans

Bacillus lentus

Bacillus licheniformis

Bacillus pumilis

Bacillus pumilus

Bacillus subtilis

Bifidobacterium adolescentis

Bifidobacterium animalis

Bifidobacterium animalis ssp. *lactis*

Bifidobacterium animalis subsp.

Bifidobacterium bifidum

Bifidobacterium breve

Bifidobacterium infantis

Bifidobacterium lactis

Bifidobacterium longum

Bifidobacterium pseudolongum

Bifidobacterium thermophilum

Brevibacterium casei

Brevibacterium linens

Candida colliculosa

Candida famata

Candida glabrata

Candida lambica

Candida milleri

Candida utilis

Candida valida

Clostridium butyricum

Clostridium sporogenes phage

Clostridium tyrobutyricum phage

Corynebacterium flavescens

Debaryomyces hansenii

Enterococcus faecium

Enterococcus mundtii

Geotrichum candidum

Hafnia alvei

Kluyveromyces lactis

Kluyveromyces marxianus

Kluyveromyces marxianus var. lactis

Kluyveromyces marxianus-fragilis

Kluyveromyces thermotolerans

Kocuria varians

Lactobacillus acidophilus

Lactobacillus alimentarius

Lactobacillus amylolyticus

Lactobacillus amylovorans

Lactobacillus brevis

Lactobacillus brevis var. lindneri

Lactobacillus buchneri

Lactobacillus bulgaricus

Lactobacillus carnis

Lactobacillus casei

Lactobacillus casei rhamnosus

Lactobacillus casei ssp. rhamnosus

Lactobacillus cellobiosus

Lactobacillus collinoides

Lactobacillus curvatus

Lactobacillus delbrueckii

Lactobacillus delbrueckii ssp. *bulgaricus*

Lactobacillus delbrueckii ssp. *lactis*

Lactobacillus farciminis

Lactobacillus helveticus

Lactobacillus jensenii

Lactobacillus johnsonii

Lactobacillus lactis ssp. *lactis*

Lactobacillus lactis ssp. *lactis* biovar *diacetylactis*

Lactobacillus leichmanii

Lactobacillus mucosae

Lactobacillus paracasei

Lactobacillus paracasei ssp. *paracasei*

Lactobacillus pentosus

Lactobacillus plantarum

Lactobacillus reuteri

Lactobacillus rhamnosus

Lactobacillus sakei

Lactobacillus sakei subsp. *sakei*

Lactobacillus salivarius

Lactobacillus sanfrancisco

Lactobacillus xylosum

Lactococcus acidophilus

Lactococcus lactis

Lactococcus lactis biovar. *diacetylactis*

Lactococcus lactis lactis

Lactococcus lactis ssp. *cremoris*

Lactococcus lactis ssp. *lactis*

Lactococcus lactis ssp. *lactis* biovar. *diacetylactis*

Lactococcus lactis ssp. *lactis* *diacetylactis*

Leuconostoc citrivorum

Leuconostoc dextranicum

Leuconostoc mesenteroides

Leuconostoc mesenteroides ssp. *cremoris*

Leuconostoc oeno

Leuconostoc pseudomesenteroides

Micrococcus varians

Oenococcus oeni

Oospora lactis (Synonym: *Geotrichum candidum*)

Pediococcus acidilactici

Pediococcus pentosaceus

Penicillium candidum

Penicillium nalgiovensis

Penicillium roqueforti

Pichia fluxuum

Propionibacterium shermanii

Propionibacterium acidipropionici

Propionibacterium acidi-propionici

Propionibacterium freudenreichii

Propionibacterium freudenreichii ssp. *shermanii*

Propionibacterium globosum

Propionibacterium shermanii

Propionibacterium sp

Rhizopus oryzae

Rhodopseudomonas palustris

Saccharomyces cerevisiae

Serratia rubidaea

Staphylococcus carnosus

Staphylococcus carnosus ssp. carnosus

Staphylococcus carnosus ssp. utilis

Staphylococcus warneri

Staphylococcus xylosus

Streptomyces griseus

Streptococcus salivarius ssp. thermophilus

Streptococcus cremoris

Streptococcus diacetylactis

Streptococcus faecium

Streptococcus lactis

Streptococcus salivarius ssp. thermophilus

Streptococcus thermophilus

Torulasporea delbrueckii

Viniflora oenos

Appendix 2. List of enzymes included in literature search

3-Phytase

6-Phytase

Acetolactate decarboxylase (alpha)

Acetylhexosaminidase (beta-L-N)

Alginate lyase

Alpha-amylase

Aminopeptidase

AMP deaminase

Amylase

Amylase (alpha)

Amylase (beta)

Arabinanase

Arabinofuranosidase

Asparaginase

Beta-1,4 glucanase cellulase

Beta-1,4 xylanase

Beta-glucanase

Carboxypeptidase (serine-type)

Catalase

Cellobiose dehydrogenase

Cellulase

Cellulase-hemicellulase complex

Cellulase-xylanase complex

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Chitinase

Cyclodextrin gluconotransferase

Dextranase

Dextranucrase

Endo-1,3(4)-beta-glucanase

Endo-1,3-beta-glucanase

Endo-1,4-beta-D-mannanase

Endo-1,4-beta-glucanase

Endo-1,4-beta-xylanase

Esterase

Ferulic acid esterase

Galactosidase (alpha)

Glucanase (beta)

Glucoamylase

Glucoamylase or Amyloglucosidase

Glucose isomerase

Glucose oxidase

Glucosidase (alpha)

Glucosidase (exo-1.3-beta)

Glucosyltransferase or Transglucosidase

Glutaminase

Hemicellulase

Hexose oxidase

Inulase

Invertase or Fructofuranosidase (beta)

Laccase

Lactase or Galactosidase (beta)

Lipase monoacylglycerol

Lipase triacylglycerol

Lipoxygenase

Lysozyme

Maltogenic amylase

Mannanase

Mannanase (endo-1.4-beta)

Pectin lyase

Pectin methylesterase or Pectinesterase

Pectinase

Pentosanase

Peroxidase

Phosphodiesterase

Phospholipase A

Phospholipase B

Phospholipase D

Phytase

Polygalacturonase or Pectinase

Protease (incl. milkclotting enzymes)

Protein glutaminase

Pullulanase

Sulfhydryl oxidase

Tannase

Transglutaminase

Urease

Xylanase

Appendix 3. Regulation of respiratory sensitization risk of enzymes and microorganisms

Introduction

From a regulatory perspective, substances or agents with an adverse effect of human health can be regulated by a number of instruments, such as packaging and labelling precautions; restriction of persons or work places; occupational exposure limits, or enforced use of protection devices. Often an initial step is a classification of substances or agents into groups of similar risk profile.

In this appendix, the regulation concerning classification and labelling of enzymes and microorganisms are discussed together with the concepts of occupational exposure limits.

EU legislation: classification

Enzymes

In terms of regulation the industrial enzymes are treated the same way as low molecular chemical substances in terms of classification and labelling. The of risk of respiratory sensitization is covered by the *COMMISSION DIRECTIVE 2001/59/EC of 6 August 2001: adapting to technical progress for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances* [196]. Here the relevant definition of the criteria that should be used for identifying the inhalation sensitising potential is described, i.e. which criteria that should be applied for evaluation of whether a substance is eligible for being labelled with R42:

3.2.7.1. Sensitization by inhalation

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Substances and preparations shall be classified as sensitising and assigned the symbol .Xn., the indication of danger .Harmful. and the risk phrase R42 in accordance with the criteria given below.

R42 May cause sensitization by inhalation

if there is evidence that the substance or preparation can induce specific respiratory hypersensitivity,

where there are positive results from appropriate animal tests, or

if the substance is an isocyanate, unless there is evidence that the specific isocyanate does not cause respiratory hypersensitivity.

Comments regarding the use of R42:

Human evidence

Evidence that the substance or preparation can induce specific respiratory hypersensitivity will normally be based on human experience. In this context hypersensitivity is normally seen as asthma, but other hypersensitivity reactions such as rhinitis and alveolitis are also considered. The condition will have the clinical character of an allergic reaction. However, immunological mechanisms do not have to be demonstrated.

When considering the evidence from human exposure, it is necessary for a decision on classification to take into account in addition to the evidence from the cases:

the size of the population exposed,

the extent of exposure.

The evidence referred to above could be:

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clinical history and data from appropriate lung function tests related to exposure to the substance, confirmed by other supportive evidence which may include:

- * a chemical structure related to substances known to cause respiratory hypersensitivity,
- * an *in-vivo* immunological test (e.g. skin prick test),
- * an *in-vitro* immunological test (e.g. serological analysis),
- * studies indicating other specific but non-immunological mechanisms of action, e.g. repeated low-level irritation, pharmacologically mediated effects, or
- * data from a positive bronchial challenge test with the substance conducted according to accepted guidelines for the determination of a specific hypersensitivity reaction.

Clinical history should include both medical and occupational history to determine a relationship between exposure to a specific substance or preparation and development of respiratory hypersensitivity.

Relevant information includes aggravating factors both in the home and workplace, the onset and progress of the disease, family history and medical history of the patient in question. The medical history should also include a note of other allergic or airway disorders from childhood, and smoking history.

The results of positive bronchial challenge tests are considered to provide sufficient evidence for classification on their own. It is however recognised that in practice many of the examinations listed above will already have been carried out.

Substances that elicit symptoms of asthma by irritation only in people with bronchial hyperreactivity should not be assigned R42.

Animal studies

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Data from tests which may be indicative of the potential of a substance or preparation to cause sensitization by inhalation in humans may include:

IgE measurements (e.g. in mice), or
specific pulmonary responses in guinea pigs.

Microorganisms

Whole microorganisms are different because they are living cells, i.e. biological agents.

The above-mentioned directive covers dangerous substances. According to the new EU Regulations on chemicals (REACH), the definitions of chemicals (substances and preparations) according to REACH are:

Substance means a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Preparation means a mixture or solution composed of two or more substances

Microorganisms such as a bacterium, a yeast cell or spores of these do not fall under the definition of a substance or a preparation according to the EU Regulations. Application of the classification criteria and labelling rules to the area of microorganisms would thus be by way of analogy with substances and preparations, since they do not seem to be covered by the Regulations mentioned above.

EU has, however, covered the occupational hazards of working with biological agents in a directive on *protection of workers from risks related to exposure to biological agents at work*

A definition of “biological agents” and “microorganisms appears in 90/679/EEC Council Directive 90/679/EEC of 26 November 1990 on the protection of workers from risks related to exposure to biological agents at work:

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Biological agents shall mean microorganisms, including those which have been genetically modified, cell cultures and human endoparasites, which may be able to provoke any infection, allergy or toxicity.

Microorganism shall mean a microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material.

In the biological agent directive [197] and the Council Directive 93/88/EEC of 12 October 1993 amending Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work (seventh individual Directive within the meaning of Article 16 (1) of Directive 89/391/EEC) it is suggested to classify biological agents in four groups and additionally mark the individual organisms with the following characteristics:

[Appendix III, Introductory note #10:] This list also gives a separate indication in cases where the biological agents are likely to cause allergic or toxic reactions, where an effective vaccine is available, or where it is advisable to keep a list of exposed workers for more than 10 years.

These indications are shown by the following letters:

A: Possible allergic effects

D: List of workers exposed to this biological agent to be kept for more than 10 years after the end of last known exposure

T: Toxin production

V: Effective vaccine available

The most recent revision, is Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC) also refers to the above classification and provides a list of microorganisms.

While there are no bacteria classified as having *possible allergic effects* this classification has been applied to 8 (groups of) fungal species:

Aspergillus fumigatus

Candida albicans

Coccidioide sinunitis

Cryptococcus neoformans var. *neofonnans* (*Filobas idiella neofonnans* var. *neofonnans*)

Cryptococcus neoformans var. *gattii* (*Filobasidiella bacillispora*)

Epidermophyton floccosum

Microsporum spp.

Penicillium marneffe

The above-mentioned list formed the basis of national lists in the UK and Germany. These are some years newer, and have been slightly revised for virus (SARS), but there has been no changes regarding (the missing) classification of bacteria as allergenic, and no further fungal species defined as allergenic. The list from Directive 2000/54/EC only include organisms in classes 2, 3 and 4, but the German list also mention of class 1 organisms [198]. Unfortunately this list does not provide information on potential sensitization. A recent guideline from Germany [199] discusses airway sensitising potential and risks of biological agents:

(4) Biologische Arbeitsstoffe mit sensibilisierender Wirkung sind in der Regel Schimmelpilze und einigen Bakterien (u.a. Actinomyceten). (p. 3)

And further

(3) Einige Bakterien (u.a. thermophile Actinomyceten) sowie Pilze und wenige Parasiten können am Arbeitsplatz allergische Atemwegserkrankungen auslösen. Auch nicht lebensfähige Bakterien, Pilze (abgestorbene Zellen, Bruchstücke oder Sporen) und Parasiten oder ihre Bestandteile (z.B. Proteine) können atemwegssensibilisierend wirken. Erfahrungsgemäß führt erst längerfristige Exposition gegenüber atemwegssensibilisierenden biologischen Arbeitsstoffen in hoher

Konzentration zu einer Sensibilisierung bis hin zu schwerwiegenden allergischen Erkrankungen. (p. 5)

Thus it is not considered a general feature of bacteria to express sensitising properties.

Also as indicated in the introduction the Regulation on (chemical) substances and preparations does not cover biological agents, thus it is not surprising that there is no mention of microorganisms in this legal complex.

In conclusion, no bacterial species *per se* have previously been considered as allergenic in the European legislation. On the other hand, individual fungi and yeasts have been recognized as allergenic in accordance with the scientific literature as discussed later.

Occupational exposure limits

Setting of occupational exposure limits

Occupational exposure limits (OELs) are concentrations of products in the air. Health-based OELs are set by a risk assessment approach, which includes evaluating all hazards (inborn toxicological properties) linked to a compound and establishing exposure-response relationships for the hazardous effects, followed by risk characterization. Concentrations below the OELs are considered to protect nearly all occupationally exposed individuals against adverse effects, although it is realized that a minor and especially sensitive part of the population may not be protected [200]. This subpopulation may include individuals highly asthmatic/allergic to the allergen of interest.

A prerequisite for establishing a health-based OEL is that adverse reactions are exposure-dependent and that there is a concentration where adverse effects no longer appear, i.e. it is possible to establish a no-observed-(adverse)-effect level (NO(A)EL) for offending effects [200]. For allergen exposures, risk assessment may be evaluated by means of the airborne concentration per m³, as with OELs. Appropriate analytical methods are prerequisites for establishing exposure-response relationships. Airborne allergens are collected on filters by means of a pump and sampling may be

in the breathing zone by a person-carried filter cassette or it may be by high volume static samplers. The filter content of allergens is then analyzed by immunochemical methods [201,202].

A risk may also be evaluated by means of the allergen concentration per gram of settled dust[203]. The idea behind this approach is that settled dust can become airborne and thus the concentration in the settled dust can be used as a proxy for the exposure. This approach has been found useful for evaluation of indoor allergen exposures from house dust mite, dog, cat, cockroach, mouse urinary protein and *Alternaria alternaria* [204].

For toxicological reactions in general, one NO(A)EL is considered for the key effect. However, for allergic reactions two phases have to be considered. First, exposure to an allergen may induce “sensitization” that implies production of specific antibodies or activated immune cells. Sensitization is not *per se* a disease. Second, “elicitation” of symptoms occurs with further exposures to the allergen at a sufficient dose [205,206]. Thus, two limits may be set, one where no sensitization is observed and another one that prevents the elicitation of allergic reactions in already sensitized individuals [203,206].

Thresholds and exposure-response relationships for airborne allergens

It may be difficult to establish thresholds for allergen exposures due to inter-individual variations in susceptibility to both sensitization and elicitation. Those may be caused by genetic differences (atopy versus non-atopy), heterogeneity of immune properties of allergens, age-dependent effects and lifestyle factors (e.g. smoking). There may also be differences between doses that induce sensitization and those that induce elicitation of symptoms [180,207]. Co-exposure to endotoxins may also play a role in development of sensitization and asthma [208]. Nevertheless, “practical” NOAELs have been proposed for several environmental and occupational allergen exposures [180,207] (Table 1).

The prerequisite of a clear exposure-response relationship has been demonstrated for several airway allergens [180,207,209]. This has been shown for indoor and outdoor allergen exposure to house dust mites, cockroaches, pets, pollen and moulds. The exposure-response relationship often shows a monotonous increase in sensitization and development of allergy with increasing allergen exposure

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[205]. However, cross-sectional studies have suggested a bell-shaped relationship, like in the case of cat allergens, where high exposure levels may induce tolerance [210]. A similar relationship has been observed in laboratory animal workers exposed to rat allergens, which may be due to a “modified T helper type 2 (Th2) response” where specific IgG4 antibodies plays a role [211]. This is heavily debated though, as a behavioral effect, i.e. “healthy petkeeper effect” cannot be totally excluded [212]. The general trend that IgE sensitization and IgE-mediated allergies increase with exposure levels has been substantiated by studies of exposure to enzymes used in the detergent industry [213,214], of exposure to natural rubber latex allergens and of exposure to flour dust as discussed below. Enzymes and natural rubber latex allergens are used as examples of highly potent allergens, where an OEL has been set for the proteolytic enzyme subtilisin. Flour dust is used as an example of how to set an OEL for a low-potent allergen.

Potency and exposure levels of allergens

The exposure-response relationships for allergens have two important features, the steepness of the relationship and the position of the exposure-response curve. Thus, different proteins have different sensitization potencies. For example, sensitization to rat urinary allergens occurs in the pg/m^3 range, sensitization to fungal α -amylase in the ng/m^3 range, whereas sensitization to wheat, pig and cow proteins occurs in the $\mu\text{g/m}^3$ range [209]. That different allergens seem to have different potencies is also deduced from environmental allergen exposures as only a limited number of allergens are of major importance in the general population [205]. Thus, the number of allergy cases in a population depends both on the potency of the allergen, the presence of adjuvants and the particle size, as well as on the specific exposure levels. In the German population in 1999, the number of occupational asthma cases caused by various exposures was in the order flour>latex>food and feed [207].

IgE-mediated allergy and hypersensitivity pneumonitis

Few OELs have been set officially for macromolecular biological compounds. They comprise OELs for the enzyme subtilisin and for flour dust. However, industry has often set its own internal

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OELs for enzyme, and OELs for enzymes have been proposed in the scientific literature with the purpose to protect against IgE-mediated allergies (type I reactions). In addition, more of these agents have an EU labelling or have warnings in OEL lists. These warnings are based on recognized hazards, i.e. when an increased incidence of allergy has been observed in an occupationally exposed population. Hypersensitivity pneumonitis (HP) is caused by immune reactions of precipitating antibodies and activated immune cells. Few cases of enzyme-induced HP have been reported. On the other hand, many cases of type I allergies to enzymes have been documented. Therefore, the critical effect is considered to be the type I allergy and thus, measures preventing the type I enzyme allergy are considered also to prevent HP. Most HP reactions appear from high exposures to airborne moulds and bacteria, where growth has been promoted by wet and humid conditions. In these cases, risk management should limit the wet and humid conditions, or otherwise limit growth of microorganisms. If not possible appropriate airway protection should be used.

Occupational exposure limit for subtilisins

The subtilisins are proteolytic enzymes derived from *Bacillus subtilis* or closely related organisms. In the early 1970s, the American Conference of Governmental Industrial Hygienists (ACGIH) established an OEL for subtilisins as a ceiling level of 60 ng/m³ of the 100% active pure enzyme [215]; a ceiling level being a concentration that should not be exceeded during any part of the working exposure [216]. Thus, for a well controlled working environment, the exposure concentration has to be considerably lower than the ceiling level in general, which is important to consider when comparing the value, for example, with an 8-hour time-weighted average OELs (Table 1).

The OEL was derived from the experiences in the surfactant industry in the late 1960s and the beginning of the 1970s. The main purpose was to minimize the potential for symptoms as sore throat, nasal congestion, cough, wheezing, allergic respiratory sensitization, and to minimize skin irritation [215]. This value is one of the lowest OELs ever established and it still applies [216]. No OEL has been set for other enzymes by ACGIH [216]. No OEL has been set for enzymes in

Germany [217], Japan [218], or by the EU Scientific Committee on Occupational Exposure Limits (SCOEL), but an OEL (Table 1) has been set for subtilisins in the UK [219].

These values are basically bypassed by the much lower exposure levels presently attended by the detergent industry. Also, the lower “in house” OELs in industry take into account that other constituents of detergents may have adjuvant effects [34,35,206]. In the near future, OELs will have to be set for many enzymes to fulfil the requirement of the new EU REACH legislation. Thus, a value has to be based on a NOAEL approach to establish the “derived no effect level” (DNEL) or, where a NOAEL cannot be established, the OEL has to be based on an acceptable risk to set a “derived minimal effect level” (DMEL)[206].

Microorganisms and occupational exposure limits

Setting and using OELs in relation to unintended development of microorganisms in naturally occurring constituents would not be possible in most cases due to the limited possibility of identifying the offending microorganisms, the limited knowledge about exposure-response relationships, and the limited possibility to implement such values in relation to risk management strategies. In these cases, the risk management strategy should directly address the appropriate prevention of the offending exposure.

Labelling

Proteins as airway allergens – hazard based warning against allergy

In the EU, harmonized classification and labelling are adopted for about 8,000 compounds

([http://www.reach-compliance.eu/english/legislation/docs/launchers/launch-annex-1-67-548-](http://www.reach-compliance.eu/english/legislation/docs/launchers/launch-annex-1-67-548-EEC.html)

[EEC.html](http://www.reach-compliance.eu/english/legislation/docs/launchers/launch-annex-1-67-548-EEC.html)), which uses a hazard-based approach. Of these compounds, very few are proteins, which are known airway allergens (Table A3.1). In the EU, hazards are described by symbols, e.g. harmful is indicated by “Xn” and an irritant is assigned the symbol “Xi”, and by risk (R) phrases

(http://ec.europa.eu/environment/chemicals/dansub/consolidated_en.htm). Thus, specific respiratory

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hypersensitivity (asthma, rhinitis and alveolitis) is indicated by the risk phase R42: May cause sensitization by inhalation. Although animal studies may be used, the evidences are mainly from human studies, where the size of the population exposed and the extent of exposure are taken into account.

For α -amylase, β -glucosidase, cellulase, exo-cellobiohydrolase and laccase, respiratory hypersensitivity was the only hazard identified. For the proteases, chymotrypsin, ficin, papain, pepsin A, rennin, subtilisin and trypsin, additionally hazards were identified as expressed from R36: Irritating to eyes, R37: Irritating to respiratory system, R38: Irritating to skin, and R41: Risk of serious damage to eyes or the combination of these sentences.

The EU classification and labelling indicates different risk management approaches by means of different safety (S) phrases as S2: Keep out of the reach of children, S21: When using do not smoke, S22: Do not breathe dust, S23: Do not breathe gas/fumes/vapour/spray (appropriate wording to be specified by the manufacturer), S24: Avoid contact with skin, S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice, S36: Wear suitable protective clothing, S37: Wear suitable gloves, S39 Wear eye/face protection, and S45: In case of accident or if you feel unwell seek medical advice immediately (show the label where possible) or their combinations (http://ec.europa.eu/environment/chemicals/dansub/consolidated_en.htm).

No account shall be taken of substances classified as harmful, corrosive or irritant if they exist as impurities or as additives, if their concentration by weight is less than 1 % and if not otherwise specified (<http://www.dehp-facts.com/upload/documents/webpage/document32.pdf>).

Hazard identifications from organizations setting occupational exposure limits are limited. In Germany, no OEL is set for the protein allergens, but hazard-based warnings are added to several proteins (Table A3.1). Thus, respiratory allergens are indicated by “Sa” or “Sah” if it is also a skin sensitizer. Only human exposure effects are accepted. Sufficient evidence is accepted if specific hyperreactivity has been observed in relation to exposure in more than one subject in at least two independent testing centres and if there is indication for an immunological mechanism. An allergic effect is also accepted from one single case report of a specific hyperreactivity of the airways or the lungs together with other sensitizing effects, e.g. a close structure-effect relationship with known airway allergens [217]. Other examples of warnings to sensitizers are included in Table 1.

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Read-across to establish allergenic properties within a certain type of enzymes may not be possible. For example, sensitization to phytase derived from *Trichoderma* and *Peniophora* species did not show cross-reactions. However, cross-reactions were observed for different types of enzymes derived from the same microorganism [118].

Overall, warning systems about sensitizing properties have to take into account that different allergens or allergenic systems can show highly different potencies and that exposures of humans need to reach a certain level before clinical effects appear. Established warning systems take into account that a certain number of observed allergies have been observed in exposed populations.

Table A3.1. Proteins as airway allergens: Labelling and occupational exposure limits

Compound	CAS-nr	Classification ^{a)}	Labelling ^{a)}	Occupational exposure limits ^{b)}
α -Amylase Other amylases	9000-90-2 ^{a)} -	R42	Xn R: 42 S: (2-)22-24-36/37	DFG: Sa Baur: LOAEL~ 0.25 ng/m ³ Sen
Bromelain juice	9001-00-7 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	DFG: Sa
β -Glucosidase?	9001-22-3 ^{a)}	R42	Xn R: 42 S: (2-)22-24-36/37	
Cellulase Other cellulases	9012-54-8 ^{a)} -	R42	Xn R: 42 S: (2-)22-24-36/37	DFG: Sa
Chymotrypsin	9004-07-3 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	

Enzymes and microorganisms as respiratory sensitizers

Exo-Cellobiohydrolase	37329-65-0 ^{a)}	R42	Xn R: 42 S: (2-)22-24-36/37	
Ficin	9001-33-6 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	
Laccase	80498-15-3 ^{a)}	R42	Xn R: 42 S: (2-)23-45	
Papain	9001-73-4 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	DFG: Sa
Pepsin A	9001-75-6 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	
Phytase	-	-	-	DFG: Sa
Proteases, not mentioned	-	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	
Proteinase, microbial neutral	9068-59-1 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	
Rennin	9001-98-3 ^{a)}	Xi; R36/37/38 R42	Xn R: 36/37/38-42 S: (2-)22-24-26-36/37	
Subtilisin	9014-01-1 ^{a, b)} (DFG) 1395-21-7 ^{b)} (DFG)	Xi; R37/38-41 R42	Xn R: 37/38-41-42 S: (2-)22-24-26-36/37/39	ACGIH: C 60 ng/m ³ DFG: Sa HSE: 40 ng/m ³ TWA Baur: LOAEL < 5 ng/m ³ Sen
Trypsin	9002-07-7 ^{a)}	Xi; R36/37/38	Xn R: 36/37/38-42 S: (2-)22-24-26-	

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		R42	36/37	
Xylanases	37278-89-0 ^b (DFG))	-	-	DFG: Sa
Flour dust (wheat, rye, barley and oats)		-	-	ACGIH: 0.5 mg/m ³ TWA SEN DECOS: LOAEL~1 mg/m ³ SCOEL: NOAEL/LOAEL~1 mg/m ³ HSE: 10mg/m ³ TWA and STEL: 30 mg/m ³ Sen Baur: NOAEL 0.5-1 mg/m ³
Ricinus protein				DFG: Sa
Soya bean constituents				DFG: Sa

- a) EU classification and labelling of dangerous substances and preparations see text.
- b) ACGIH [216]: “C” indicates a concentration that should not be exceeded during any part of the working exposure, “TWA” the 8-hour Time Weighted Average, and “SEN” that it is a sensitizer; DFG [217]: “Sa” indicates a respiratory allergen; DECOS [220]; HSE [219]: the short-term exposure limit is indicated by “STEL” (15-min reference period) and sensitization by “Sen”; Baur [207]: “NOAEL” and “LOAEL” for humans, “Sen” indicates sensitization and “A” asthma. For further explanations see text.

Basis for labelling and when to set OELs for macromolecular biological compounds

A warning, including a labelling, is relevant when a group of individuals is at a substantiated risk for developing diseases. To be meaningful, a warning about airway allergy or other immunological airway reactions should be based on the incidence of sensitization or the incidence of disease in exposed populations. The highest incidence is expected where process concentrations are highest, which may be in relation to production or downstream use of pure compounds. Health monitoring at such workplaces is therefore especially important for hazard identification.

In general, OELs are set to allow exposure control in relation to production of compounds as well as control of exposures in downstream users. Setting an OEL requires hazard identification as for labelling, but also a quantitative risk assessment. This requires that the adverse effect is exposure-dependent and that a level can be set where a potential risk is so low that it is of no concern. Thus, a quantitative relationship about the exposure-response relationship has to be established. Appropriate analytical methods must be available for this purpose, but also for the control of compliance with the established OEL. As for flour dust, the overall dust concentration in the air may sometimes be used as proxy for the airborne allergen concentration, but in most cases, the analytical methods have to address the air concentration of the allergenic protein itself.

At present, very few OELs exist for the macromolecular biological compounds. In the near future, it is expected DNELs or DMELs will be set for several industrial enzymes according the European REACH regulation and thus providing a new type of OELs. For enzymes, the key effect seems to be IgE-mediated allergy.

Appendix 4. Companies that have been interviewed

Chr. Hansen A/S

The company was founded in 1874, and has been handling dried bacteria cultures for 30 years. They produce 2400 tons of cultures per year in 12 factories, and handle about 100 different species in the process. This accounts for about 50% of the world production. They currently have 700 employees active in the production chain.

Novozymes A/S

This company produces enzymes for industrial purposes, both from yeast and bacteria and claim to hold more than 50% of the global market. It has been operating at an industrial scale since the 1940s and employs workers potentially exposed to the enzymes are in the 1000 range, each potentially exposed to several products. Novozymes A/S monitors allergies among workers thru screening programs, cap-tests, and self-reporting, and also conducts a significant amount of research in the field of allergy.

Questions to the companies

Technical part:

- Which categories of enzyme and/or microbial cultures does your company produce and/or use?
- Of these categories, which volume does your company handle on a yearly or monthly basis?

- How many workers are exposed to each of those categories?
- When did your company start to handle those enzymes or cultures?

Occupational part:

- Is your company monitoring allergies among workers and/or customers?
- Are you aware of allergy cases (within the company or with your customers or end-users) related to some of the enzymes or cultures that your company handles?
- Have your company developed any special formulations that reduce the risk of exposure to cultures or enzymes?
- Can you pinpoint any products, processes or unit operations that are related to either particularly high risk for sensitization or particularly low risk for sensitization?
- Does your company take specific measures for working with some of those cultures or enzymes (e.g.: personal protection equipment) within the company or for customers or end-users

Toxicological part:

- Has your work with enzymes or cultures given you an impression on the dose-response-relationships in allergy development?
- Are you able to rank the allergy risk between individual processes or products?
- Are your company using any methods for predicting a possible risk of sensitization for any particular product?

Glossary/Abbreviations

AD: atopic dermatitis

AMFEP: Association of Manufacturers and Formulators of Enzyme Products

CI: confidence interval

CT: computed tomography

DC: dendritic cell

DMEL: derived minimal effect level

DNEL: derived no effect level

EFSA: European Food Safety Authority

HP: hypersensitivity pneumonitis

ICU: intensive care unit

NO(A)EL: no-observed-(adverse)-effect level

OEL: occupational exposure level

OVA: ovalbumine

QPS: qualified presumption of safety

RSV: respiratory syncytial virus

SPT: skin-prick test

TARC: thymus and activation-regulated chemokine