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Differential effects of synbiotic delivery route (Feed, water, combined) in broilers challenged with *Salmonella* Infantis

Victoria Drauch [a,1,*], Mahdi Ghanbari [b,1], Nicole Reisinger [b], Michaela Mohnl [b], Claudia Hess [a,2], Michael Hess [a]

[a] Clinic for Poultry and Fish Medicine, University of Veterinary Medicine Vienna, Vienna, Austria
[b] DSM-Firmenich, Animal Nutrition and Health R&D Center, Tulln, Austria

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar Infantis (*S.* Infantis) presents a persistent and multi-drug-resistant threat to poultry production, highlighting the need for effective control strategies. This study evaluated the impact of a *S.* Infantis infection in broiler chickens across various parameters, including organ colonization, gut microbiota, and immune function. We also assessed the mitigation potential of a synbiotic, multispecies feed additive, administered via three routes applicable for the field: feed only, drinking water only, and a combination of both. Our results demonstrated that the combined administration route yielded notably positive effects on several parameters, followed by the drinking-water only administration. This approach resulted in significant improvements in gut microbiota health, characterized by increased levels of beneficial microbes such as *Lactobacillus, Ligilactobacillus*, and *Butyricicoccus*, and a decrease in potentially harmful genera from the Proteobacteria phylum. Reduction of *S.* Infantis load was observed in caecum, ileum, and spleen over time albeit shedding was not influenced. The drinking water-only administration showed a significant reduction of *S.* Infantis colonization in the caecum on the last sampling day. Immune response analysis indicated no significant differences in serum antibody levels between control and treatment groups. These findings underscore the impact of both combined and drinking water-only synbiotic, multispecies feed additive administration on the gut microbiota and a possible route for reducing *S.* Infantis in poultry production. The obtained data provide valuable guidance for optimizing synbiotic use in commercial poultry management, enabling enhanced pathogen control and improved gut health.

Introduction

Salmonella enterica subspecies *enterica* (*S.*) causes the second most common reported zoonosis in humans – salmonellosis. One of the serovars, *S.* Infantis, has experienced an emerging increase within the last two decades in broiler production, and it is now the most common isolated serovar in broiler samples in the European Union and the fourth most common serovar in humans (EFSA, 2024). This increase in *S.* Infantis is associated with the presence of a megaplasmid known as the 'plasmid of emerging Salmonella Infantis' (pESI) (Aviv et al., 2014) as well as enhanced persistence on farms (Pate et al., 2019; Newton et al., 2020; Zeng et al., 2021), resistance to disinfectants (Drauch et al., 2020), increased biofilm production (Koyuncu et al., 2013; Moraes et al., 2018, 2019), the presence of antimicrobial resistance genes (Pate et al., 2019; Nagy et al., 2020) and greater susceptibility in fast-growing broilers (Drauch et al., 2022). Therefore, it is essential to find effective prevention methods to reduce the spread of *S.* Infantis (Drauch et al., 2022; Montoro-Dasi et al., 2023).

Modulation of the chicken intestinal microbiome via natural and non-antibiotic solutions such as probiotics (Oh et al., 2017; El-Shall et al., 2020; El-Sharkawy et al., 2020; Khan et al., 2020; Shanmugasundaram et al., 2020), prebiotics (Adhikari et al., 2018; El-Shall et al., 2020; Wu et al., 2020) and synbiotics (Luoma et al., 2017; Villagrán-de la Mora et al., 2020) has emerged as an intriguing strategy to prevent

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* Corresponding author.
E-mail address: victoria.drauch@vetmeduni.ac.at (V. Drauch).

[1] These authors contributed equally to this work.
[2] Present address: LVA, Magdeburggasse 10, 3400 Klosterneuburg, Austria.

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intestinal infections by *Salmonella* during the production cycle, and thus promoting the overall health and performance of chickens in the production setting. For example, studies on *Lactobacillus (L.) fermentum* have shown promising results in sustaining intestinal health in chickens characterized by improved height of intestinal villi (destroyed by *Salmonella*) throughout the gut and, thus, an improvement in the surface area for nutrient absorption (Šefcová et al., 2023). Furthermore, specific probiotic strains like L. *salivarius* 16/c6 have been effective in inhibiting *Salmonella* adhesion (El Hage et al., 2022), while products such as Broilact® (Orion Corporation, Espoo, Finland) have shown efficacy in preventing *S.* Infantis colonization in newly hatched chicks (Schneitz et al., 2016). Although previous studies have demonstrated the potential benefits of probiotics and synbiotics in poultry, there is a noticeable gap to elucidate functional components considering the variation between products. Understanding the effectiveness of synbiotics against *S.* Infantis is crucial for developing targeted interventions in poultry production. Recognizing this, we conducted a comprehensive study to investigate the impacts of administering a synbiotic supplement containing *Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis, Limosilactobacillus reuteri, Ligilactobacillus salivarius,* and the soluble fiber inulin in three different application routes to broilers being challenged with *S.* Infantis. Shedding and colonization behavior of *S.* Infantis were assessed as well as serological markers, alterations in gut microbiota, and changes in blood biomarkers to evaluate the impact of synbiotic intervention. From a practical perspective understanding the differential effects of each administration method on the gut microbiota and overall poultry health is essential for developing tailored strategies that maximize efficacy. By evaluating the efficacy of the synbiotic through multiple application routes, our study aims to provide valuable insights into their optimal utilization for controlling *S.* Infantis in poultry production. Ultimately, this research not only contributes to a better understanding of bacterial interaction in the gut but also offers practical solutions that can be implemented to enhance food safety and promote sustainable poultry production practices.

Material and methods

Birds and housing

Approval for the animal trial was given by the institutional ethics committee and licensed by the national authority according to the Austrian law for animal experiments (license number GZ.: 2022-0.474.400). 125 one-day old commercial ROSS 308 broilers (Brueterei Schulz, Lassnitzhoehe) were randomly divided into 5 groups resulting into 25 birds per group. Each group was housed in a separated isolator (Montair HM2500, Montair Environmental Solutions B.V., Kronenberg, The Netherlands) and water and feed were given *ad libitum*. The feed composition and calculated nutrients of broiler diet used in the study is detailed in Table 1, at day 14 of age feed was changed from Phase 1 to Phase 2 feed. On the day of arrival chicks were tagged subcutaneously with an individual number tag (SwiftagTM, Heartland Animal Health Inc. Fair Play, Missouri, USA). The trial was terminated when the birds reached 35 days of age.

Preparation of Salmonella Infantis inoculum

For the infection study a well-defined and fully sequenced *S.* Infantis strain (accession no: SAMN19328299) was used. The strain was isolated from a broiler environmental sample in Austria and contains a pESI-like megaplasmid, which includes genes such as irp2, ipf, klf, ccdB/ccdA, and two plasmid-encoded fimbrial operons, pef and sta (Drauch et al., 2021). A bacterial suspension of the strain was cultured overnight at 37°C in Luria-Bertani-Broth (LB, Invitrogen, Vienna, Austria) using a shaking incubator (250 rpm). Colony-forming-unit (CFU) count was determined by plating serial dilutions (1:10) on MacConkey agar (Bertoni, Vienna, Austria) in duplicates, and the mean value was calculated.

Table 1
Detailed feed composition and calculated nutrients of broiler diet used in the study.

Ingredient	Unit	Phase 1 (Days 0-14)	Phase 2 (Days 14-35)
Corn	%	56.40	60.95
Soya bean meal hp	%	30.20	26.10
Full fat soya	%	6.00	6.00
Calcium carbonate	%	1.38	1.32
Mono calcium phosphate	%	0.87	0.54
Fat powder	%	1.60	2.00
Sunflower oil	%	1.00	1.00
Potato protein	%	0.70	0.60
Lignocellulose	%	0.65	0.50
Sodium chloride	%	0.32	0.32
Sodium sulfate	%	0.07	0.05
L-Lysine	%	0.30	0.18
DL-Methionine	%	0.25	0.26
L-Threonine	%	0.09	0.03
Vitamin premix	%	0.08	0.08
Trace element premix	%	0.08	0.08
Calculated nutrients per kg as fed			
Metabolizable energy (Poultry)	MJ	12.6	12.9
Dry matter	%	88	88
Crude protein	%	21.6	19.8
Crude fiber	%	3.2	3.1
Crude fat	%	6.6	7
Crude ash	%	5.4	4.8
Ca	%	0.9	0.8
P	%	0.6	0.5
Na	%	0.2	0.2
Mg	%	0.2	0.2
K	%	0.9	0.9
Cl	%	0.2	0.2
Lysine	%	1.4	1.2
Methionine	%	0.6	0.6
Cyst(e)ine (as cysteic acid)	%	0.3	0.3
Threonine	%	0.9	0.9
Tryptophan	%	0.3	0.3
Vit. A	I.U.	10000	10000
Vit. D3	I.U.	5000	5000
Vit. E	mg	60	60
Vit. K3	mg	4.0	4.0
Vit. B1	mg	3.0	3.0
Vit. B2	mg	8.0	8.0
Vit. B6	mg	5.0	5.0
Vit. B12	mcg	20	20
Nicotinic Acid	mg	70	70
Pantothenic acid	mg	20	20
Choline chloride	mg	2000	2000
Folic Acid	mcg	2200	2200
Biotin	mcg	220	220
Iron	mg	15	15
Copper	mg	15	15
Zinc	mg	100	100
Manganese	mg	110	110
Iodine	mg	1.2	1.2
Selenium	mg	0.5	0.5

The bacterial suspensions were then washed and re-suspended in phosphate-buffered saline (PBS, GIBCO) and the infection dose was adjusted to a concentration of 10^8 CFU/ml.

Synbiotic composition

The synbiotic, multispecies feed additive (PoultryStar®, Biomin GmbH, Herzogenburg, Austria) contained inulin (prebiotic) and the probiotic strains *Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis, Limosilactobacillus reuteri* and, *Ligilactobacillus salivarius*. All strains were isolated from diverse intestinal compartments from healthy chickens (Klose et al., 2006).

Experimental design and sampling

The experimental setup is explained in detail in Fig. 1. Briefly, 125-day-old chickens were assigned to five groups. Three of these groups were administered the synbiotic supplement using three different application routes: in feed and drinking water (SynFW_SI), in drinking water only (SynW_SI), and in feed only (SynF_SI). The synbiotic was administered in both the feed and drinking water to maintain the desired probiotic concentrations throughout the trial. In the feed, it was included at a dosage of 1 kg per ton, supplying probiotic strains at a level of 10^8 CFU/kg, with *ad libitum* access provided to all groups throughout the trial. For the drinking water, the synbiotic premixture was freshly prepared on each day of application. During the first week of life, a dosage of 0.6 g per liter of drinking water was administered on the day of arrival and on days 1, 2, 5, 6, and 7. From day 13 onward, the dosage was reduced to 0.3 g per liter on days 13, 14, 15, 21, 22, 28, 29, 34, and 35, supplying probiotic strains at a level of 10^9 CFU/L water. The synbiotic was specifically formulated to provide effective concentrations in both feed and drinking water. To ensure that the synbiotic preparations met the target concentrations, microbial analyses were conducted on the synbiotic premixtures and feed samples. Total lactic acid bacteria counts were determined on MRS Agar (Oxoid) following ISO 15214 (ISO 15214, 1998). The results were as follows: 8.52 ± 0.08 log10 CFU/g for the synbiotic premixture for feed application, 9.74 ± 0.03 log10 CFU/g for the synbiotic premixture for water application, and 5.51 ± 0.13 log10 CFU/g for feed, all of which were in good agreement with the target concentrations. The remaining two groups served as controls: a positive control group that received only the *S.* Infantis infection (SI) and a negative control group that received neither the synbiotic nor infection (control).

On day 7 of age, chicks in four groups (SynFW_SI, SynW_SI, SynF_SI, and SI) were infected with 1 ml of *S.* Infantis (10^8 CFU/ml) via crop tube, while birds in the negative control group (control) were administered 1 ml of phosphate-buffered saline (PBS, GIBCO, Paisley, UK). The clinical condition of the birds was monitored twice daily and scored from 0 (no clinical symptoms) to 4 (poor health conditions). Housing system parameters (temperature, humidity, airflow, and air pressure) in the isolators were monitored daily.

Cloacal swabs (FloqSwabs, Copan, Italy) were collected from birds on the day of arrival and twice weekly following infection to analyze shedding behavior and weekly by 16S-rDNA-based microbiota analysis. Duplicate cloacal swabs were obtained to assess the shedding of *S.* Infantis. One swab was directly streaked onto xylose-lysine-deoxycholate (XLD) agar (Merck, Vienna, Austria) and incubated aerobically at 37°C for 24 h. The second swab was stored at 4°C to enable an enrichment procedure, according to EN ISO 6579-1:2017 (EN ISO 6579-1, 2017) , in case the direct plating yielded a negative result. Results were classified as "high shedding" if direct plating detected *S.* Infantis, "low shedding" if *S.* Infantis was detected only after enrichment, and "negative" if neither method detected *S.* Infantis.

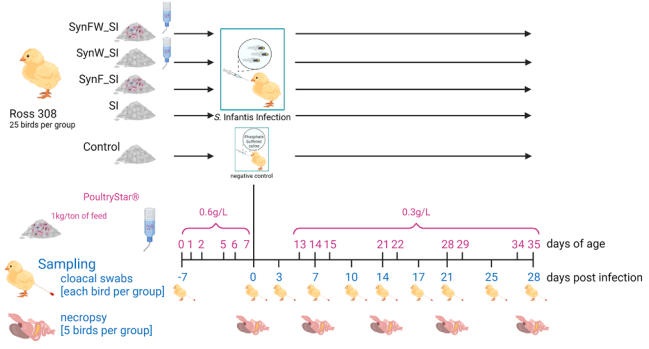

Fig. 1. Experimental design of the animal trial administering a synbiotic supplement via three routes: feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI). Control groups included a positive control (SI) and a negative control (control). Broilers were infected with *Salmonella* Infantis on day 7. Sampling involved cloacal swabs from all birds and necropsy of 5 birds per group on each killing day.

The bacterial load in internal organs (caecum, liver, and spleen) was quantified by conducting CFU counts. Euthanasia was performed before infection on day 7 in 5 birds per group, and at 7, 14, 21, and 28 days post-infection (dpi) for the same number. Sedation was achieved via intramuscular injection of a 1:1 mixture of Sedaxylan® (20 mg/ml, Dechra Pharmaceuticals, Dornbirn, Austria) and Narketan® (100 mg/ml, Vetoquinol, Vienna, Austria), followed by euthanasia via jugular vein bleeding. Necropsies followed a standardized protocol, recording bodyweight, liver, and spleen weights, and collecting caecum, liver, and spleen samples for bacteriological analysis. Liver samples were also taken for histological examination, and blood was collected for serological testing. Each organ sample (1 g) was homogenized (ULTRA TURRAX T 10 basic, IKA, Staufen, Germany) in 1 ml of PBS, and serial dilutions (1:10) of the homogenates were prepared in PBS and plated in duplicate on XLD agar. Plates were incubated at 37°C for 24 h to count single colonies, with the average CFU/g calculated per organ. Original homogenates were kept at 4°C to enable the EN ISO 6579-1:2017 enrichment procedure if direct plating was negative.

Histology

The liver of each bird was formalin fixed and embedded in paraffin. Samples were cut into 5 µm sections with a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany) and stained with hematoxylin and eosin (HE).

Serology

Blood was collected during necropsy from the *V. jugularis*. For antibody analysis, 2 ml Eppendorf tubes (Roth, Germany) were filled with at least 1 ml of blood, centrifuged at 4000 g for 12 min to separate the serum, which was then frozen at −20°C. An indirect ELISA was performed as described previously by Drauch et al. (2022).

Determination of blood chemistry and acute phase protein alpha-1 acid glycoprotein (AGP)

Plasma was analyzed for aspartate aminotransferase (AST), bile acids (BA), creatine kinase (CK), uric acid (UA), glucose (GLU), phosphorus (PHOS), total protein (TP), albumin (ALB), and globulin (GLOB) using the VETSCAN ® VS2 Chemistry Analyzer with the VetScan® Avian Reptilian Profile Plus rotors (Zoetis, Vienna, Austria), according to the manufacturer's recommendations. Plasma concentration of the acute-phase protein alpha-1-acid glycoprotein (AGP) was measured using commercially available ELISA kits (Life Diagnostics, West Chester, PA, USA). The assay was performed according to the manufacturer's instructions.

Cloacal swab DNA extraction, 16S rRNA gene sequencing and bioinformatic analysis

Five birds per group were randomly selected each week for microbiota analysis via cloacal swabs throughout the trial. Cloacal swabs were taken in triplicates from these birds on a weekly basis. To preserve the material, the tips of the three cloacal swabs were cut off and stored in an Eppendorf tube filled with RNALater (Thermo Fisher Scientific, United States). DNA extraction from the cloacal swabs was performed using the QIAamp PowerFecal Pro DNA Kit (Qiagen) according to the manufacturer's instructions. Subsequently, library preparation was conducted using the 341F-805R 16S primer set (341F: 5′-CCTACGGGNGGCWGCAG-3′; 805R: 5′-ACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013), followed by sequencing on an Illumina MiSeq platform using v2

chemistry (Microsynth Austria GmbH). The 16S rRNA gene data analysis was carried out using the dada2 pipeline (Callahan et al., 2016) with paired end fastq files. Trimming and truncation of reads were automatically executed using the FIGARO tool (Sasada et al., 2020), which required all reads to have uniform lengths. The 'Length of the sample reads' parameter was set to 250 base pairs. SILVA 138 database used for taxonomical annotation of the Amplicon Sequence Variants (ASV) (Quast et al., 2012). All reads with abundance below 0.1 % were removed from the downstream analysis.

Statistical analysis

Data was analyzed with R (R Core Team 4.2.2). For assessment of clinical condition, bacteriological as well as serological data, three packages (tidyverse, ggpubr and nortest) were used. Exploratory Data Analysis was performed, and normality was assessed with the Anderson-Darling-Test. For normal distributed data a t-test was used to test for significant differences. In case normal distribution was rejected, log transformation was performed. To analyze qualitative data such as shedding behaviour and organ colonization a generalized linear model was implemented with a specification for binominal distribution. Quantitative data (bodyweight, weight of liver, weight of spleen, CFU Count and OD Values) were analyzed using a Linear Model.

For Bioinformatic analysis of microbiota data the variance explained by each variable included in the trial was quantified by omnibus testing on Bray–Curtis dissimilarity matrices derived from 16S amplicon sequencing data, using PERMANOVA with the adonis2 function in the R package Vegan (Dixon, 2003). Ordination was performed with log-transformed normalized reads on two dimensions using Local Fisher Discriminant Analysis (LFDA). Linear regression analysis was performed considering random differences between the treatment groups at the first sampling as covariate terms in the model if the richness, neutral and inverse Simpson diversity were affected by the different treatments. Neutral diversity refers to the Hill number of $q = 1$ (equivalent to the Shannon diversity or exponential to the Shannon index), which weighs bacteria according to their relative abundances (Alberdi et al., 2019). Log-fold changes in abundance between groups were determined by a negative binomial generalized linear model using DESeq2 version 1.34.0. (Love et al., 2014) in R, considering the statistical significance at BH-corrected $P \leq 0.10$. Data from Blood chemistry and AGP analysis were tested for normality using the Shapiro–Wilk test. If data were normally distributed, ANOVA was conducted, followed by Bonferroni post-hoc tests. For non-normally distributed data, a Kruskal–Wallis test was applied. Statistical significance was determined at $p < 0.05$. In all statistical analyses, the animal was considered as the experimental unit.

Results

Clinical symptoms, necropsy, bodyweight, and organ weight

No clinical symptoms were observed, and no gross pathological lesions were found during necropsy. Bodyweight increased significantly ($p < 0.05$) at 7 dpi in the groups administered the synbiotic via drinking water, either alone (SynW_SI) or in combination with feed (SynFW_SI) (Figure in Supplementary File, Fig. S1). No differences were observed in the spleen-bodyweight ratio between groups. However, when examining the liver-bodyweight ratio, the SynFW_SI group showed a significant increase at 28 dpi ($p < 0.05$) compared to the control (Figure in Supplementary File, Fig. S2).

Bacteriology

All samples of the negative control as well as samples from all groups before infection were negative for *S.* Infantis.

Shedding. Birds shedding *S.* Infantis were observed in all groups starting from 3 dpi, which was the first sampling point after infection.

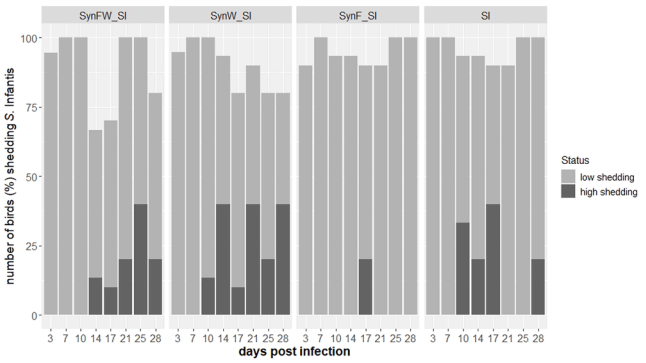

Fig. 2. Percentage of birds shedding *Salmonella* Infantis across treatment groups: synbiotic administered in feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI), as well as positive control (SI) and negative control (control). Shedding is categorized as high (detected via direct plating of cloacal swabs) or low (detected only after enrichment).

Fig. 2 illustrates the shedding behavior of birds infected with *S.* Infantis over time, assessed via cloacal swabs. The results are categorized by the percentage of birds exhibiting low-shedding, high-shedding, or negative results. Primarily, low shedding was observed across all four groups infected with *S.* Infantis, with the SynF_SI group showing high shedding in birds only at 17 dpi. The highest shedding was observed in the challenge group (SI), while birds administered the synbiotic in feed and water (SynFW_SI) exhibited the least shedding of *S.* Infantis, followed by the group administered the synbiotic in the drinking water only (SynW_SI).

Organ colonization of *Salmonella* Infantis. Qualitative analysis of organ colonization was ranked within three categories: positive via direct plating, positive via enrichment procedure or negative. The results are illustrated in Fig. 3 and showed that the highest colonization in all four infected groups was found in the caecum, shortly after the ileum, followed by the spleen and the least colonization noticed in the liver. As presented in Fig. 3, the results of the qualitative analysis were similar between the four groups and no statistical significance was detected. However, in SynFW_SI the ileum colonization was found to be the least compared to the other groups, giving mainly positive results following enrichment procedure, whereas the colonization in the liver was highest in this group.

For all samples which were positive via direct plating a CFU count was performed, and thus quantitative analysis of *S.* Infantis colonization was feasible and results are given in Fig. 4. On the first day of organ

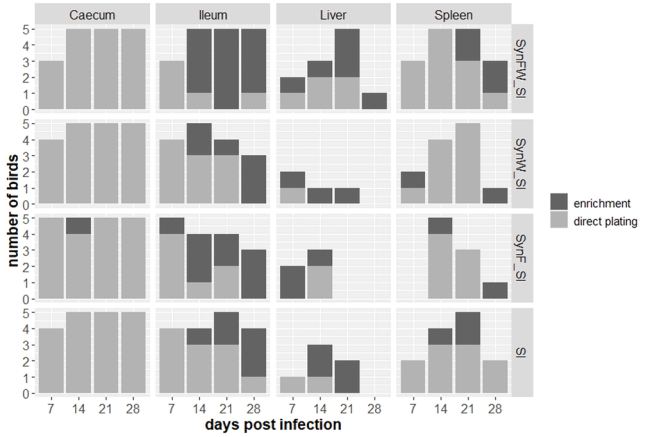

Fig. 3. Qualitative analysis of *Salmonella* Infantis organ colonization in only infected group (SI) and in three different synbiotic application groups all infected as well: synbiotic in feed and water (SynFW_SI), synbiotic in water (SynW_SI), synbiotic in feed (SynF_SI).

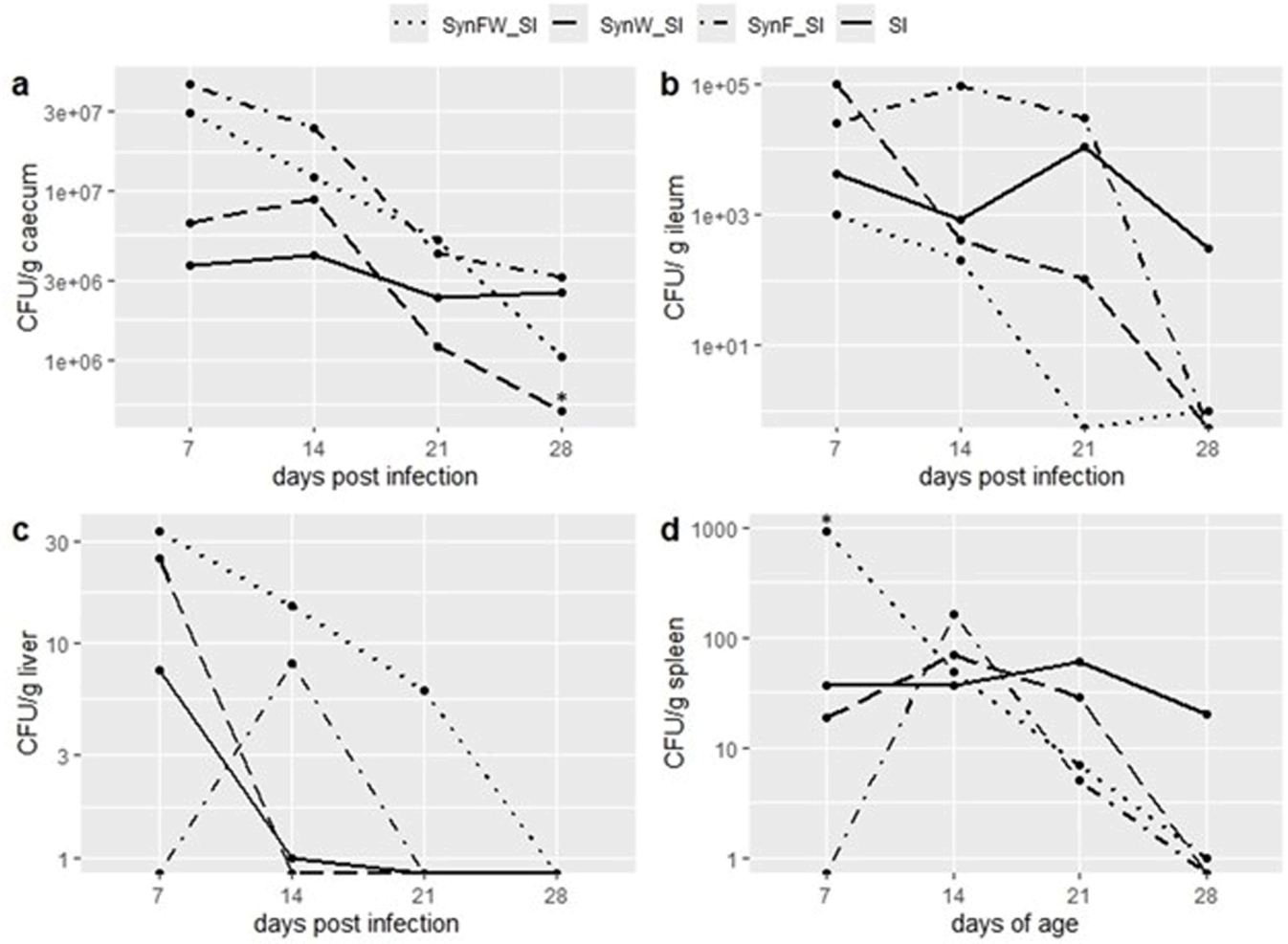

Fig. 4. Quantitative analysis of *Salmonella* Infantis colonization measured as Colony Forming Unit (CFU) count per gram of organ across treatment groups: synbiotic administered in feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI), as well as positive control (SI) and negative control (control). Statistical comparison was tested against CFU data from only infected birds (SI) and marked with * for significant differences ($p < 0.05$).

sampling (7 dpi) in 8 out of 12 cases (including different administrations as well as organs) birds administered synbiotic either way - showed an increased *S*. Infantis colonization compared to the only infected group with a statistically significant result ($p < 0.05$) in the group administered the synbiotic via feed and water (SynFW_SI). However, *S*. Infantis colonization decreased in all groups administered the synbiotic till the last sampling day (28 dpi), whereas the colonization of the only infected group stayed consistent in caecum, ileum and spleen but decreased in liver where the slowest decline was noticed in SynFW_SI group. In ileum and spleen all three groups administered the synbiotic showed a decrease of colonization. In caecum it was the case for two (SynFW_SI and SynW_SI) out of the three groups and in SynW_SI this decrease was statistically significant ($p < 0.05$).

Histology

Although a difference in the liver-bodyweight ratio was observed, no morphological differences were found between groups in the liver upon examination using HE (Hematoxylin and Eosin) staining.

Serology

Antibody development measured with an in-house ELISA is outlined in Fig. 5. A significant increase in antibody levels was observed in all infected groups from 21 dpi onwards compared to the negative control group (control). However, no significant differences were found

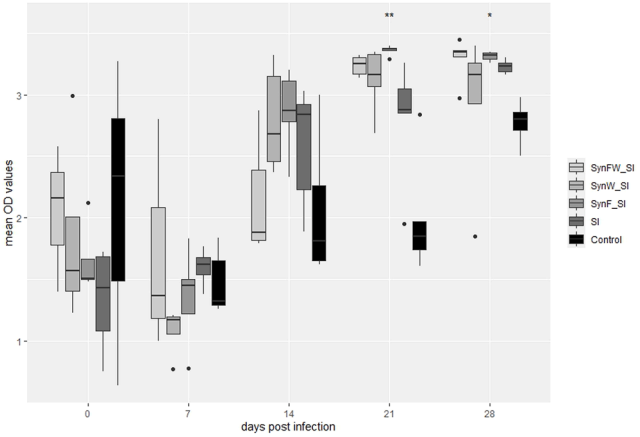

Fig. 5. Mean optical densities indicating antibody development against *Salmonella* Infantis measured with an in-house ELISA across treatment groups: synbiotic administered in feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI), as well as positive control (SI) and negative control (control). Statistically significant differences compared to the control group are marked with * ($p < 0.05$) or ** ($p < 0.01$).

between the infected groups.

Blood chemistry and acute phase protein alpha-1 acid glycoprotein (AGP)

Mean blood chemistry results including SD of each group per sampling day are summarized in Table 2. Bile acid concentration was excluded from statistical analysis as the majority of samples were below the detection limit. No differences were found in creatine kinase and albumin at any timepoint ($p > 0.05$, Table in Supplementary File, Table S2). SynFW_SI showed a lower AST activity compared to all other groups on 7 dpi ($p < 0.05$). The uric acid concentration of SynF_SI was increased on 28 dpi compared to SynW_SI ($p < 0.05$). The glucose concentration in blood increased in all four *Salmonella* infected groups compared to the uninfected group at 7, 14 and 21 dpi and at 7 dpi there was an increase in SynW_SI group compared to the control ($p < 0.05$). The phosphorus concentration at 7 dpi was decreased in all four infected groups compared to the control ($p < 0.05$) and increased at 28 dpi in SynWF_SI compared to the control, SI and SynW_SI ($p < 0.05$). The total protein concentration increased in the SynWF_SI group compared to the the SynW_SI group on 28 dpi ($p < 0.05$). In addition, the globulin concentration was increased in SynWF_SI and SynF_SI compared to SynW_SI at 28 dpi ($p < 0.05$). No difference in the AGP levels between treatments was found at any timepoint ($p > 0.05$; Figure in Supplementary File, Fig. S3).

Impact of Salmonella Infantis and the synbiotic on the broiler gut microbiota

16S rRNA gene amplicon sequencing of 146 cloacal swab samples (four samples excluded due to extremely low DNA yield) generated over 5 million paired-end sequence reads, with a mean of 38,000 reads per sample (see Supplementary File 1 for detailed information on the samples, including library size).

The PERMANOVA analysis of microbiota data highlighted key drivers of variation (Fig. 6A). The age of the birds (sampling time points: −7, 0, 7, 14, 21, and 28 dpi) emerged as the primary factor, explaining 21.5 % of the total variation. Additionally, the impact of *S.* Infantis challenge and synbiotic application collectively accounted for 12.7 % of the variation, indicating significant effects on microbial communities. Intriguingly, the interaction between treatment and age was substantial, explaining 49.1 % of the variation, underscoring the dynamic response of microbiota to interventions over time. Although individual differences among birds contributed to 23.3 % of the variation, this was not statistically significant, yet underscores the inherent variability in microbiota composition across individual chickens.

Focusing on the effect of treatments on the gut microbiota variation, Pairwise PERMANOVA comparisons showed significant differences between various groups. The *S.* Infantis challenge alone (SI) was associated with a notable shift in gut microbiota composition compared to the control group ($R^2 = 0.044$, $P < 0.05$, adjusted-$P > 0.05$). Although this difference was not statistically significant after adjusting for multiple comparisons, the trend shows a potential impact of *Salmonella* infection

Table 2
Mean AST, UA, GLU, PHOS, TP, and GLOB levels in broilers from the negative control group (Control), infected with *Salmonella* Infantis on day 7 without synbiotic supplementation (SI), and with synbiotic supplementation via water (SynW_SI), via feed (SynF_SI), or via both water and feed (SynFW_SI).

	Aspartate Aminotransferase (AST) U/L				**Uric Acid (UA) mg/dL**			
	7 dpi	**14 dpi**	**21 dpi**	**28 dpi**	**7 dpi**	**14 dpi**	**21 dpi**	28 dpi
Control	240.6[a] ± 20.28	169.6 ± 27.15	197.6 ± 41.30	202.0 ± 26.13	14.8 ± 3,31	12.6 ± 2.19	11.5 ± 1.55	9.7[ab] ± 3.13
SI	201.5[a] ±42.35	161.0 ± 24.42	154.8 ± 54.80	193.4 ± 69.03	15.8 ± 3.79	11.3 2.07	9.6 ± 3.00	11.0[ab] ± 3.88
SynW_SI	197.3[a] ± 9.74	194.2 ± 22.31	239.0 ± 30.07	226.2 ± 71.53	16.7 ± 3.08	11.5 ± 2.09	10.6 ± 1.56	7.7[a] ± 1.16
SynF_SI	184.5[a] ±10.28	184.8 ± 17.12	250.0 ± 66.22	263.6 ± 91.22	15.7 ± 3.08	10.6 ± 1.91	11.1 ± 3.00	13.5[b] ± 2.65
SynWF_SI	95.0[b] ± 64.05	177.2 ± 18.27	216.8 ± 70.86	352.8 ± 133.8	9.9 ± 1.25	11.1 ± 2.45	9.9 ± 2.40	10.9[ab] ± 2.00
P-Value	0.0005	0.1906	0.0974	0.1527	0.0703	0.6797	0.8061	0.0425
	Glucose (GLU) mg/dL				**Phosphorous (PHOS) mg/dL**			
	7 dpi	**14 dpi**	**21 dpi**	**28 dpi**	**7 dpi**	**14 dpi**	**21 dpi**	**28 dpi**
Control	227.8[a] ± 18.46	382.8 ± 117.2	332.6 ± 126.6	425.8 ± 164.2	11.7[a] ± 1.03	11.5 ± 2.44	11.5 ± 2.01	10.4[a] ± 10.2
SI	437.5[ab] ± 153.1	409.0 ± 149.4	368.8 ± 150.8	502.6 ± 131.6	8.1[b] ± 1.23	9.0 ± 1.29	10.2 ± 1.82	12.3[a] ± 3.34
SynW_SI	469.0[b] ± 150.8	422.0 ± 85.71	382.0 ± 100.7	290.2 ± 45.11	8.7[b] ± 1.07	13.0 ± 2.96	13.8 ± 3.18	10.8[a] ± 0.85
SynF_SI	294.4[ab] ± 83.27	341.8 ± 70.31	371.8 ± 156.2	522.8 ± 152.0	8.5[b] ± 1.15	10.9 ± 2.24	13.2 ± 1.46	13.5[ab] ± 1.69
SynWF_SI	291.0[ab] ± 37.16	472.0 ± 131.4	472.8 ± 179.8	454.0 ± 132.1	8.7[b] ± 0.25	11.3 ± 0.57	13.5 ± 3.51	16.4[b] ± 1.80
P-Value	0.0412	0.4913	0.6352	0.0824	0.0014	0.0785	0.1529	0.0006
	Total Protein (TP) g/dL				**Globulin (GLOB) g/dL**			
	7 dpi	**14 dpi**	**21 dpi**	**28 dpi**	**7 dpi**	**14 dpi**	**21 dpi**	**28 dpi**
Control	2.73 ± 0.15	2.78 ± 0.08	2.80 ± 0.23	2.98[ab] ± 0.11	0.20 ± 0.26	0.50 ± 0.7	0.34 ± 0.18	0.60[ab] ± 0.08
SI	2.83 ± 0.17	2.84 ± 0.05	2.80 ± 0.21	3.00[ab] ± 0.42	0.25 ± 0.17	0.52 ± 0.11	0.44 ± 0.19	0.64[ab] ± 0.26
SynW_SI	2.83 ± 010	2.90 ± 0.37	3.08 ± 0.26	2.80[a] ± 0.10	0.25 ± 0.06	0.46 ± 0.17	0.54 ± 0.15	0.46[a] ± 0.11
SynF_SI	2.58 ± 0.13	2.92 ± 0.08	3.20 ± 0.32	3.20[ab] ± 0.12	0.34 ± 0.06	0.50 ± 0.12	0.60 ± 0.14	0.83[b] ± 0.05
SynWF_SI	2.67 ± 0.21	2.62 ± 0.16	3.02 ± 0.24	3.40[b] ± 0.29	0.40 ± 0.00	0.38 ± 0.11	0.60 ± 0.19	0.82[b] ± 019
P-Value	0.1269	0.0587	0.0987	0.0105	0.3306	0.3913	0.1648	0.0136

[ab] Superscripts indicate significant difference ($p < 0.05$).

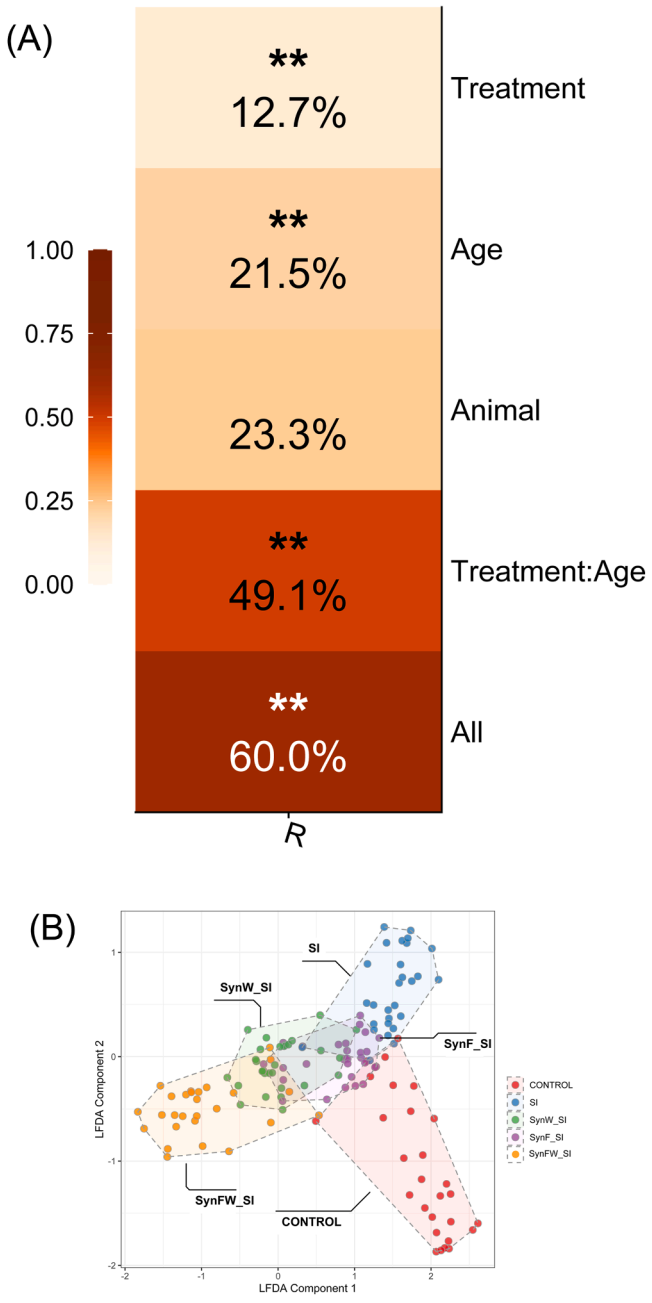

Fig. 6. (A) The percentage of variation in gut microbiota evaluated by different influencing factors. Stars show FDR-corrected statistical significance (FDR $^{**}P \leq 0.01$). Variance is estimated for each feature independently, except for "All," for which all metadata are included in the model. (B) LFDA analysis showing taxonomical clustering changes across treatment groups: synbiotic administered in feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI), as well as positive control (SI) and negative control (control).

on the gut microbiota which was evident throughout the study period (7, 14, 21, 28 dpi) and indicates that *S.* Infantis leads to an initially disrupted gut microbiota as well as long-term changes.

More significant changes were found for *Salmonella* infected birds that were administered the synbiotic. The group receiving both *S.* Infantis challenge and the synbiotic applications via feed and water (SynFW_SI) exhibited the most substantial differences in microbiota composition compared to the control group ($R^2 = 0.169$, adjusted-$P < 0.05$) over time. The SynFW_SI group differed significantly from the SI group ($R^2 = 0.142$, adjusted-$P < 0.05$), indicating that the synbiotic application had a distinct effect beyond the *Salmonella* challenge alone. The impact of different routes of synbiotic application on the microbiome was further examined by comparing groups that received synbiotic via feed (SynF_SI), water (SynW_SI), and both (SynFW_SI). The SynFW_SI group differed significantly from the SynF_SI group (synbiotic via feed only) ($R^2 = 0.101$, adjusted-$P < 0.05$) but not from SynW_SI group, suggesting that the combination of synbiotic via feed and/or water had a more pronounced effect than application by feed alone. The results showed significant differences between the control group and both the SynF_SI group ($R^2 = 0.087$, adjusted-$P < 0.05$) and the SynW_SI group ($R^2 = 0.085$, adjusted-$P < 0.05$). Direct comparisons between SynF_SI (synbiotic via feed) and SynW_SI (synbiotic via water) showed a marginal difference ($R^2 = 0.049$, adjusted-$P = 0.06$), suggesting that while both routes of administration had a significant impact on the gut microbiota, the effects were slightly different. Further, LFDA Analysis highlighted distinct clustering patterns corresponding to different treatment groups, providing further evidence of significant alterations in chicken gut microbiota composition due to *S.* Infantis and synbiotic interventions (Fig. 6B).

The effects of different treatments on three alpha diversity metrics are shown in Fig. 7. Overall, the control group exhibited relatively stable diversity across the three indices over time, although a marginal impact was noted on 21 dpi for Observed ($P = 0.06$) and Neutral diversity ($P = 0.06$) indices. In contrast, the *S.* Infantis challenge notably impacted microbial diversity, particularly evident in the Observed metric. Significant changes were observed at 7 dpi ($P < 0.05$), with marginal impacts noted for 14 dpi ($P = 0.08$) and 21 dpi ($P = 0.05$). The neutral diversity and Inverse Simpson for the *S.* Infantis group did not show significant changes over time. The synbiotic treatments varied in their impact on the microbial diversity, depending on the route of administration. Among these treatments, the SynW_SI group (synbiotic administered via water) demonstrated the most robust and sustained effects, particularly regarding neutral diversity and Observed richness. Significant increases were observed in the neutral diversity Index at 7 dpi ($P < 0.05$) and 14 dpi ($P < 0.05$). Additionally, there were marginal changes in Observed richness at 7 dpi ($P = 0.09$) and significant increases at 14 dpi ($P < 0.05$). The Inverse Simpson Index also showed a significant increase at 7 dpi ($P < 0.05$), indicating a pronounced early effect on evenness. In contrast, the SynFW_SI group (synbiotic delivered via both feed and water) initially displayed significant change in neutral diversity at 7 dpi ($P < 0.05$) and Observed richness at 7 dpi ($P < 0.05$), but it did not maintain this significance at 21 dpi. However, notable increases in Observed richness were evident at 28 dpi ($P < 0.05$), suggesting a more prolonged impact. Notably, there were no significant changes in the Inverse Simpson Index for this group across time points, suggesting a lack of effect on evenness. Meanwhile, the SynF_SI group (synbiotic administered solely via feed) exhibited a more modest influence on microbial diversity, with significant increases in Shannon diversity only at 7 dpi ($P < 0.05$) and at 28 dpi ($P < 0.05$). The Observed richness showed a slight effect at 7 dpi ($P < 0.05$), but there were no significant changes in the Inverse Simpson Index until 28 dpi ($P < 0.05$), indicating a delayed effect on evenness.

Taxonomic profiling of the cloacal samples in terms of the most abundant microbial genera over the course of the study is shown in Fig. 8A. In total, 1,573 different microbial ASV belonging to 50 genera were observed, of which species belonging to Firmicutes and Proteobacteria were the most dominant members of the cecal microbiota (Fig. 8A). The top 10 genera identified were *Escherichia-Shigella, Lactobacillus*, [Ruminococcus] torques group, *Enterococcus, Romboutsia, Ligilactobacillus, Clostridium* sensu stricto 1, *Proteus, Turicibacter*, and *Terrisporobacter*.

Differential abundance analysis using the DESeq2 tool revealed significant shifts in microbial genera in response to various treatments over time (Fig. 8B; Supplementary File 2). The bar plot illustrates the number of significantly affected microbial taxa across different treatment groups at multiple time points (−7, 0, 7, 14, 21, 28 dpi). In the SI group the number of affected microbial taxa remained relatively moderate

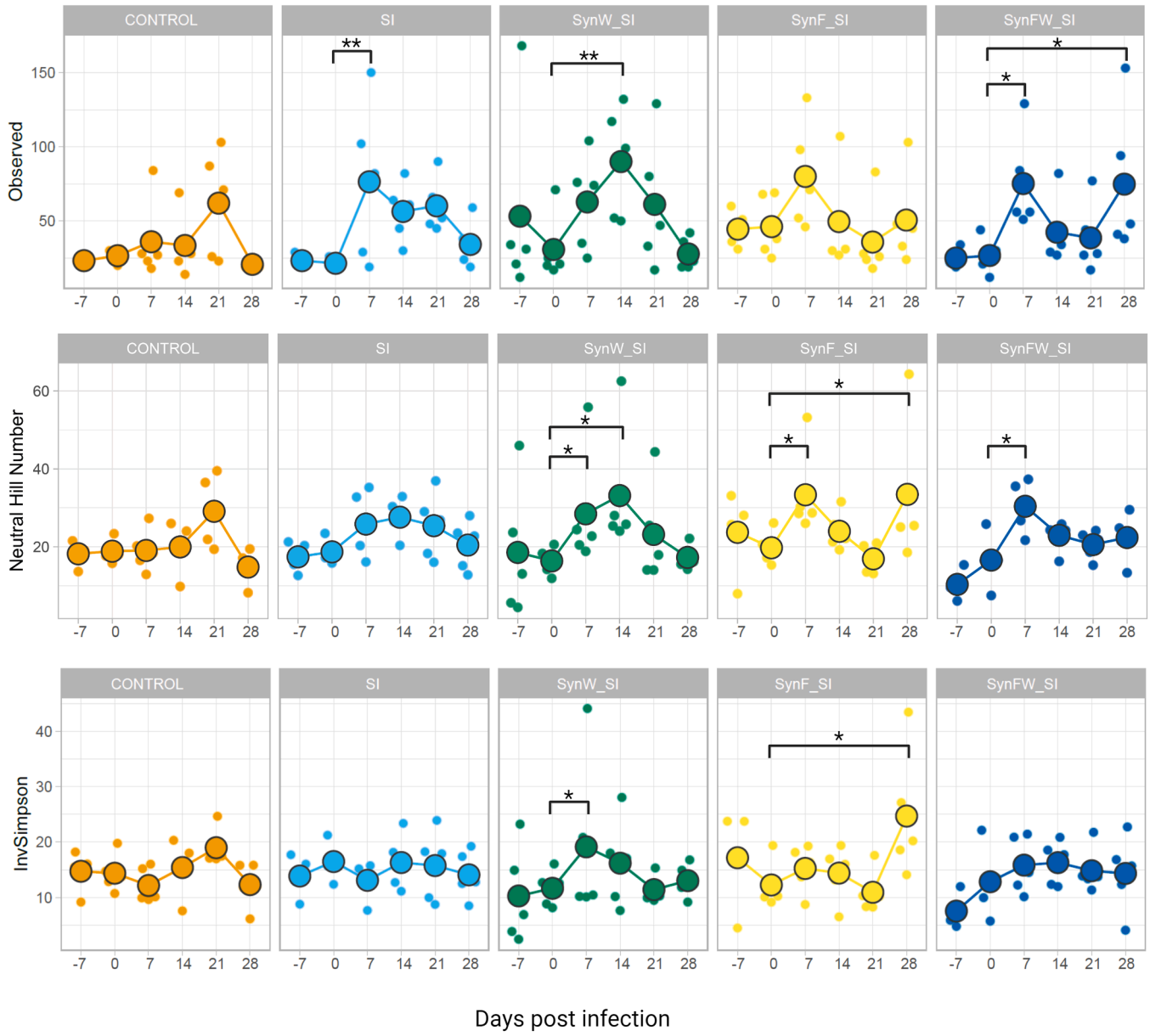

Fig. 7. Temporal variation in microbial alpha diversity indices across treatment groups: synbiotic administered in feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI), as well as positive control (SI) and negative control (control). Data are based on cloacal swab samples collected at different sampling days. Each point represents the diversity value for an individual replicate within a treatment group, while larger circles indicate the mean value for that group at each time point. Statistical significance is denoted by ** ($P < 0.01$), * ($P < 0.05$), and (*) (P between 0.05 and 0.09).

throughout the study period. Furthermore, in the SI group a notable reduction in the abundance of *Romboutsia* and *Turicibacter* was observed, indicating a significant shift in the microbial ecosystem. Conversely, there was a significant increase in *Clostridium* sensu stricto 1 as well as *Escherichia* especially on 7 and 14 dpi. In contrast, the SynW_SI group displayed significant increases in several taxa, including *Romboutsia, Ligilactobacillus*, and *Lactobacillus*. This group, which received synbiotic through drinking water, exhibited a substantial and progressive increase in the number of affected microbial taxa. By 28 dpi, this group showed the highest number of impacted taxa compared to other groups. This indicated that the synbiotic administration via water significantly modulated the gut microbiota, particularly when supplied for a longer time. For the SynF_SI group, where synbiotic was administered via feed, a steady increase in the number of affected microbial taxa was observed. However, the total number of affected taxa remained lower compared to that of the SynW_SI group, indicating that synbiotic delivery through feed also altered the gut microbiota, but to a lesser extent than the water delivery. The SynF_SI group had a notable enrichment of *Turicibacter*, HT002 and *Ligilactobacillus* (both belonging to the *Lactobacillaceae* family), similar to that of the SynW_SI group, but with a higher presence of *Turicibacter* and HT002, which were not as prominent in the SynW_SI group.

The SynFW_SI group demonstrated a significant impact on the microbial species from early on, with a large number of affected genera at 7 dpi, which continued to increase over time. On 28 dpi, this group had one of the highest counts of affected microbial taxa, second only to the SynW_SI group, indicating a synergistic effect of the combined synbiotic delivery routes in modulating the gut microbiota. Notably, there were marked increases in the abundance of genera such as [*Eubacterium*] hallii group, [*Ruminococcus*] torques group, *Butyricicoccus, Lactobacillus* and *Ligilactobacillus, Colidextribacter* and *Intestinimonas* (all of which belong to the Firmicutes phylum) and *Alistipes*, a genus within the

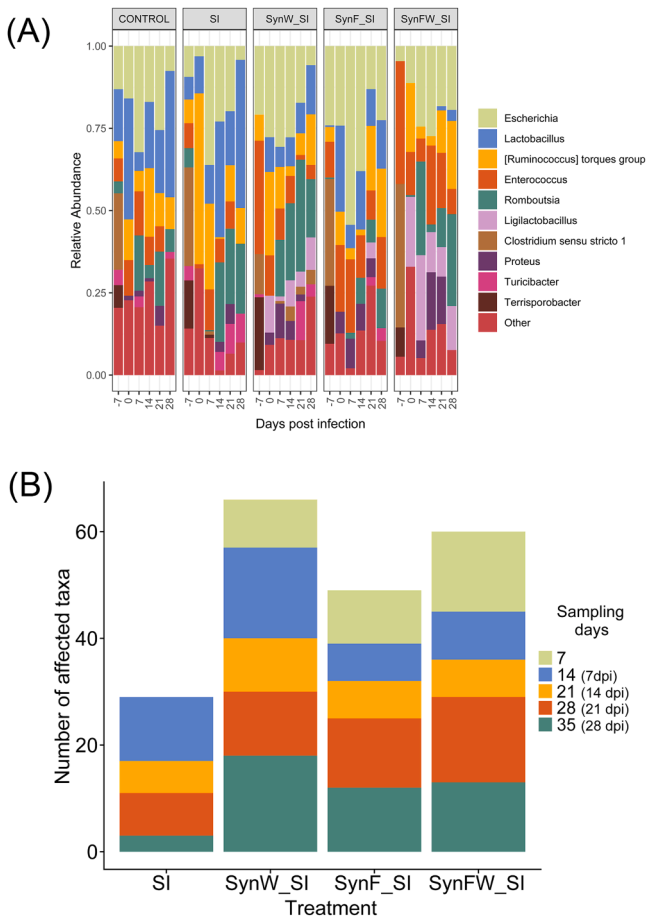

Fig. 8. A) The 10 most abundant taxa in the bacterial communities at the genus level, according to SILVA analysis, across treatment groups: synbiotic administered in feed and water (SynFW_SI), water only (SynW_SI), and feed only (SynF_SI), as well as positive control (SI) and negative control (control). B) The number of affected taxa in chicken cloacal swab samples based on differential abundance analysis for each treatment group.

Bacteroidota phylum. Conversely, the SynFW_SI group showed a significant decrease in potentially harmful genera associated with the Proteobacteria phylum such as *Escherichia, Klebsiella, Proteus*, and *Cosenzaea*.

Discussion

The consistent high prevalence of *S*. Infantis poses an ongoing risk for human health. The combination of increased environmental tenacity with the rise of multi-drug resistant isolates presents a growing public health hazard (Drauch et al., 2022). As control strategies are remaining limited so far, one option to reduce food-borne pathogens in broilers is the use of nutritional additives as pre-, pro- or synbiotics. Studies between 2012 and 2022 were reviewed recently by Mekonnen et al. (2024) showing that most studies tested probiotics, followed by prebiotics and only a small number evaluated synbiotics. The most common application route is in feed, while administration via the drinking water is used rarely and combined application via both, feed and in water, was evaluated in only one intervention study (Mekonnen et al., 2024). Thus, we conducted for the first time an infection study in broilers focusing on the colonization behaviour of *S*. Infantis and its effects on microbiota changes, in response to a synbiotic applied through three different routes – via feed only, drinking water only and feed and drinking water in combination.

In our study an increase of bodyweight due to synbiotic administration was noticed at 7 dpi in two out of three synbiotic groups. Common in these two groups was the administration of the synbiotic via the drinking water, either solely (SynW_SI) or in combination of synbiotic in feed (SynW_SI). Regarding liver-bodyweight ratio, a significant increase was present at 28 dpi in the group administered synbiotic in combination of feed and drinking water (SynFW_SI). Reports about body and organ weight of broilers fed with nutritional additives are diverging (Olnood et al., 2015; Soumeh et al., 2021; Mohammed et al., 2022). However, a meta-analysis by Sjofjan et al. (2021) evaluated 225 treatments of probiotics on growth performance as well as relative organ weight and was able to find a significant increase of both.

The shedding of *S*. Infantis was reduced in birds receiving the synbiotic supplementation, with fewer birds tested positive for *S*. Infantis in cloacal swabs. However, this reduction was not statistically significant compared to that of the infected control group. This finding contrasts with other studies where probiotics significantly reduced the shedding patterns of different *Salmonella* serovars, such as *S*. Enteritidis (Olsen et al., 2022; Kerek et al., 2023), *S*. Heidelberg (Knap et al., 2011), and naturally occurring *Salmonella* spp. colonization (Tabashsum et al., 2020). In terms of colonization patterns in the caecal content, prior studies have shown a significant reduction in *S*. Enteritidis colonization when using synbiotic (Luoma et al., 2017) and probiotic treatments (Pascual et al., 1999; Olsen et al., 2022). Similarly, probiotic treatments have demonstrated significant decreases in caecal colonization by *S*. Heidelberg and naturally occurring *Salmonella* spp. infections (Knap et al., 2011; Park et al., 2014; Tabashsum et al., 2020). In our study, the colonization of *S*. Infantis in the caecum was significantly decreased by 28 days post-infection (dpi) when the synbiotic was administered via drinking water (SynW_SI). Colonization in the ileum and spleen also showed a decreasing trend over time in treated birds compared to untreated, but infected controls, although these reductions were not statistically significant. The discrepancies between our findings and those of previous studies may be attributed to variations in *in vivo* models, chicken breed differences, or the virulence of the specific *Salmonella* strains used. Notably, our study applied a direct oral infection with *S*. Infantis, whereas other studies used a seeder model, resulting in an undefined infection dose for sentinel birds (Knap et al., 2011; Luoma et al., 2017). Additionally, it has been shown that the infection dynamics of *S*. Infantis varied among different chicken lines; in particular, fast-growing broilers were more susceptible to *S*. Infantis colonization than layer type chickens (Drauch et al., 2022). This suggests that studies conducted in layer chickens are likely to show different results from those in broilers (Pascual et al., 1999; Luoma et al., 2017; Kerek et al., 2023). An *in vitro* study demonstrated a strong inhibitory effect of probiotics on *S*. Infantis, though it noted that this effect depends on the probiotic strain used (Dos Reis et al., 2022). The higher pathogenic potential of *S*. Infantis strains carrying the pESI megaplasmid, similar to the strain used in our study, may help explain the observed discrepancies, as these strains are associated with enhanced adhesion and invasion capabilities (Aviv et al., 2014; Drauch et al., 2021). Interestingly, at 7 dpi, the colonization rate of *S*. Infantis was higher in birds receiving the synbiotic supplementation compared to those of the infected control group (SI). Lawhon et al. (2002) reported that low concentrations of short-chain fatty acids (SCFAs), such as those produced by the synbiotic used in our study, can actually promote the growth of *S*. Typhimurium. However, their findings also suggest that higher SCFA levels can ultimately inhibit colonization—a trend that aligns with our observations of a time-dependent decrease in *S*. Infantis colonization in birds administered the synbiotic.

In the present infection study, analysis of the immune response measured by serum IgY levels against *S*. Infantis revealed an increased antibody response in infected birds compared to that of non-infected controls, confirming successful infection. However, no significant differences in IgY levels were observed between the infected control and treatment groups. Similarly, Šefcová et al. (2023) reported elevated IgM levels in the serum of birds co-infected with *S*. Infantis and *Lactobacilli*,

although IgA levels remained unchanged.

To further assess the impact of the *S.* Infantis infection on broiler health, we analyzed selected biochemical blood parameters, including the acute phase protein AGP as a marker for inflammation and stress. As expected, the infection did not affect most of the measured parameters when comparing *S.* Infantis versus the negative control group. This aligns with the observation that *S.* Infantis had no impact on bodyweight at any time point and no pathological condition. Additionally, at 7 dpi, the combined administration of the synbiotic via feed and water resulted in significantly lower AST levels compared to all other groups. AST is a critical biochemical marker used to detect liver and skeletal muscle damage as well as inflammation, and it plays an essential role in regulating intracellular Ca^{2+} levels (Gowda et al., 2009). Moreover, AST is associated with the neurotransmitter serotonin, which affects neurological functions related to stress and behavior. The observed reduction in AST levels and its potential positive impact on stress and behavior are consistent with findings from Mohammed et al. (2021), who demonstrated that probiotics could reduce fear responses and stress in heat-stressed broilers. This is further supported by the fact that the bodyweight was as well increased in the group which received the synbiotic combined, via feed and water. On 28 dpi, differences related to the application route of the synbiotic were observed in uric acid, total protein, and globulin levels. However, these differences were not significant, when compared to those of the negative control and *S*. Infantis challenge groups. Furthermore, AGP was selected as a moderate acute phase protein, known to be an indicator of inflammation and stress in broilers (Cray et al., 2009). However, AGP levels were not affected by either the challenge nor the synbiotic treatment, further confirming that the *S*. Infantis challenge did not induce an inflammatory response in the birds. The only parameter affected by the challenge was the phosphorus level on 7 dpi. All infected groups, regardless of synbiotic supplementation, exhibited lower phosphorus levels compared to those of the negative control. Later on, at 28 dpi, only the combined synbiotic group showed an increase in phosphorus levels compared to both the negative control and challenge control as well as the synbiotic water group. Blood phosphorus levels can be influenced by a variety of factors, including dietary phosphorus intake, vitamin D3 levels, renal function, environmental stress, and feed additives. Probiotics may impact blood phosphorus by enhancing nutrient absorption, improving mineral metabolism, boosting immune function, modulating gut microbiota, and reduction of pathogens (Mountzouris et al., 2007).

Our investigation into the effects of a synbiotic supplementation on the gut microbiota in broiler chickens challenged with *S.* Infantis sheds light on the potential for mitigating the impact of this pathogen through microbial modulation. The *Salmonella* challenge alone induced notable shifts in gut microbiota composition and structure, as evidenced by changes in alpha and beta diversity metrics, as well as differential abundance analysis, indicating its disruptive effect on the microbial community. Interestingly, these changes persisted over time, suggesting a long-term impact of *Salmonella* infection on gut microbiota which has already been shown for other *Salmonella* serovars. Azcarate-Peril et al. (2018) reported sustained changes of the fecal microbiota of commercial white Leghorn chicks induced by a wild-type *S*. Typhimurium serovar 4, [5],12:r:− (Nalr) strain over 4 weeks. Khan et al. (2020) investigated the dynamics of gut microbiota in laying chickens during *Salmonella* infection and reported long-term effects of *S*. Typhimurium up to 12 weeks after infection on the fecal microbiota. A significant alteration in the overall caecal microbial community following infection with *S*. Typhimurium and *S*. Enteritidis have been observed in broilers (Robinson et al., 2022) as well as in layer chicken (Mon et al., 2015, 2020).

Examining the effects of a synbiotic supplementation in our study revealed significant alterations in the gut microbiota composition and biodiversity, particularly when the synbiotic was administered via the water route, alone or in combination with feed (SynW_SI and SynFW_SI), which resulted in the most pronounced and sustained modifications in microbial communities compared to the only infected group. These two groups showed a lower number of birds shedding *S.* Infantis and a decreased *S.* Infantis colonization in the caecum at 28 dpi compared to that of the challenge control, and after administrating the synbiotic via water this decrease was even statistically significant.

The synbiotic treatments were associated with an increase in beneficial microbial genera such as *Lactobacillus, Ligilactobacillus, Butyricicoccus* and *Lachnoclostridium,* known for their roles in maintaining gut health and inhibiting pathogen colonization and can possibly explain the shedding and colonization behaviour of *S.* Infantis. These findings correspond with those from previous studies that have highlighted the beneficial impact of synbiotics on the chicken gut microbiota (Brugaletta et al., 2020; Gyawali et al., 2022; Khan et al., 2020; Prentza et al., 2022; Śliżewska et al., 2020; Temmerman et al., 2022). Mountzouris et al. (2007, 2010) demonstrated that the same synbiotic, multispecies supplement administered via feed or water that was used in the current study, enhanced the growth performance and gut health of broilers by promoting beneficial bacteria from *Lactobacillus* and *Bifidobacterium* and reducing enteric pathogens in the gut of broiler chicken. Similar results were also reported by Mohammed et al. (2019; 2022) in broilers which received the synbiotic, multispecies product via feed application reared under heat stress. Brugaletta et al. (2020) reported that double supplementation of the synbiotic, multispecies supplement via gel droplets as well as feed application effectively increased the abundance of beneficial gut microbes belonging to *Lactobacillus, Bifidobacterium, Ruminococcus, Collinsella*, and *Blautia* in the caecal microbiota of broiler chickens.

A reduction in potentially harmful genera from Proteobacteria phylum, including *Escherichia* and *Proteus*, was found in the synbiotic-treated groups, particularly in the SynFW_SI group. This is consistent with other findings (Mountzouris et al., 2009; Markazi et al., 2018; Roth et al., 2019; Shanmugasundaram et al., 2019a; Mohammed et al., 2022; Temmerman et al., 2022), showing that synbiotic, multispecies feed additives administration via feed and/or water could suppress the abundance of pathogenic bacteria such as *S*. Enteritidis, commensal *E. coli* and avian pathogenic *E. coli* by outcompeting them for nutrients and through the production of antimicrobial substances (Klose et al., 2006; Markowiak et al., 2018; Shanmugasundaram et al., 2019b; Lambo et al., 2021). Klose et al. (2006) found that the probiotic species in the synbiotic, multispecies product inhibited key pathogens like *Salmonella, E. coli, Clostridium perfringens*, and *Campylobacter jejuni* via metabolic by-products such as volatile and organic acids. Additionally, *E. faecium* and *P. acidilactici* produce bacteriocins that inhibit both gram-positive and gram-negative bacteria (Jamuna et al., 2004). Shanmugasundaram et al. (2019a, 2019b) further demonstrated that supernatants from these species effectively inhibited *S.* Enteritidis *in vitro*, with *in vivo* studies confirming their competitive advantage in the gut environment. Overall, these findings underscore the potential of synbiotic supplementation to modulate the gut microbiota and the influence on *S.* Infantis colonization. The combined water-feed administration route followed by application via drinking water only turned out to be most effective, likely due to synergistic effects of synbiotic provided through both, feed and water. This may ensure more consistent and widespread colonization of beneficial bacteria throughout the gut compared to the feed only application.

Despite the promising implications of our findings, several limitations should be considered. The controlled experimental conditions may not fully reflect commercial poultry production settings, limiting the generalizability of our results. A seeder bird model, for example, could help in simulating commercial poultry farming environments. Additionally, our study focused on a single strain of *S.* Infantis, and the effects of synbiotic on other *Salmonella* serotypes or bacterial pathogens were not explored. Furthermore, while this study highlights the potential of synbiotics in controlling *S.* Infantis colonization, it is important to acknowledge that controlling *S.* Infantis requires a multifactorial approach albeit epidemiological data point towards the difficulties. Anyhow, complementary (and emerging) strategies such as

bacteriophage therapies and vaccinations of broilers, both not licensed on the market for *S*. Infantis, together with effective hygiene measures need special attention (Montoro-Dasi et al., 2023; Sáenz et al., 2022; Sevilla-Navarro et al., 2020, 2024a, 2024b). Future research should evaluate the revealed data under field production conditions, investigating the efficacy of synbiotics against a broader range of pathogens, and exploring the integration of multifactorial strategies for enhanced control of *S*. Infantis.

In conclusion, our study demonstrated that administration of a specific and well characterized synbiotic, multispecies supplement significantly modulated the gut microbiota in broilers. Comparing three different application routes showed that combining feed and water administration as well as water only administration had more pronounced effects than administration via the feed only. This was shown by the reduction of harmful genera from Proteobacteria phylum as well as a reduction of colonization of *S*. Infantis in caecum, ileum, and spleen. However, the observed reduction was less pronounced compared to that of studies focusing on *S*. Enteritidis *S*. Heidelberg or natural *Salmonella* colonization. This difference could be attributed to the unique characteristics of the used *S*. Infantis strain harboring pESI which is known to possess increased virulence and resistance factors. The findings of this study suggest approaches to improve poultry health and reduce production costs, while also providing insights into the specific characteristics and challenges of the emerging pathogen *S*. Infantis.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Victoria Drauch reports financial support was provided by Horizon Europe. Mahdi Ghanbari reports a relationship with DSM-Firmenich Animal Nutrition & Health that includes: employment. Nicole Reisinger reports a relationship with DSM-Firmenich Animal Nutrition & Health that includes: employment. Michaela Mohnl reports a relationship with DSM-Firmenich Animal Nutrition & Health that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104890.

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