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Effects of a Multi-genus Synbiotic (PoultryStar® sol) on Gut Health and Performance of Broiler Breeders

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ABSTRACT

In recent years, a rising interest has been directed towards the use of nutraceuticals in the zootechnical sector, including probiotics, prebiotics, and synbiotics, as a way to support production efficiency and cope with the increasing limitations to the use of antibiotics. In poultry, however, most studies on these products have been conducted on broilers, while less information is available on their benefits to other productive categories. The present field study aimed to assess the effects of a multi-species synbiotic product (PoultryStar® sol) on the gut health and productive performance of broiler breeders. A total of 24761 day-old Ross 308 parent stock chicks were acquired from a single hatchery and placed on the same farm. Female chicks were divided into three groups and raised in different houses (A, B, and C), in which males were introduced at the age of mating and followed until 40 weeks of age. The synbiotic was provided by drinking water to the flocks in houses A and B, while house C was kept as control. Following the manufacturer's guidelines, the product was administered intermittently once every two weeks, except in the first and the twenty-first week when it was supplied for three consecutive days. Data on performance parameters, egg quality traits, bacterial enteritis scoring, intestinal morphometry, and histopathology were recorded, and the caecal content was collected at 15, 25, and 40 weeks of age to investigate the intestinal microbiota using high-throughput next-generation sequencing. Synbiotic-treated hens showed significantly higher survivability during production compared to the control group. No clear differences were observed between treated and control chickens in terms of egg production and quality, and the effect of the synbiotic on weight gain also appeared limited. From 25 weeks onwards, synbiotic-treated chickens scored better in terms of macroscopical lesions and had longer intestinal villi. Significant differences in crypt length and histopathological lesions were also found at multiple sampling points. A treatment effect on caecal bacterial composition was detected with a differential abundance of *Gastranaerophilales*, *Lachnospiraceae*, *Helicobacter*, *Ruminococcaceae*, and *Clostridia*, among others. Taken together, obtained results support the beneficial effects of the intermittent administration of the synbiotic product PoultryStar® sol on the gut health of broiler breeders.

Keywords: Broiler breeder, Gastrointestinal health, Histopathology, Microbiota, Synbiotic

INTRODUCTION

The poultry industry is a crucial source of high-quality protein worldwide, with 199 million tonnes of chicken meat produced in 2020 (more than any other meat type) and egg production also accounting for 86 million tonnes (FAOSTAT, 2022). The unceasing growth of the sector is built upon production efficiency, pursued through genetic selection and rigorous health, nutrition, and production

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management. These measures became even more important in recent years due to the emergence of significant new challenges to the profitability and sustainability of the poultry supply chain (Mottet and Tempio, 2017; Hafez et al., 2020).

One of the key areas of interest for the poultry industry is the optimum utilization of available feed ingredients and improvements in nutrient availability (Carré et al., 2008). The intestinal health of poultry plays a role not only in the uptake of nutrients, but also in many aspects of physiology and immune response, with broad implications for animal wellbeing, production efficiency, food safety, and environmental impact (Oviedo-Rondon, 2019). Chicken gut microbiota is known to play a role in the modulation of the host's physiological functions and homeostasis, mainly through the competitive exclusion of detrimental microorganisms and pathogens (Diaz Carrasco et al., 2019). The application of 16S rRNA gene sequencing also revealed the association between enteric dysbiosis and diseases in poultry (Yang et al., 2022). For these reasons, and to cope with the increasing restrictions on the use of antibiotics, a rising interest is paid to nutraceuticals, which are seen as a potential alternative to support production performance (Alagawany et al., 2021). In particular, an ever-growing literature has been produced on probiotics, and their combinations, defined as synbiotics (Awad et al., 2009; Madej et al., 2016; Alagawany et al., 2021).

The efficacy of synbiotics relies on a synergistic effect between probiotics and prebiotics, selectively favoring the survival, implantation, and growth of beneficial bacteria populations in the gut (Awad et al., 2009; Babazadeh et al., 2011; Papatsiros et al., 2013; Nikpiran et al., 2013; Vahdatpour and Babazadeh, 2016; Alizadeh et al., 2017; Syed et al., 2020). Their capacity to improve body weight (BW) gain and feed efficiency (Mousavi et al., 2015; Luoma et al., 2017; Kridtayopas et al., 2019), modulate the immune system and stimulate the development of the gut-associated lymphoid tissue (GALT) and other lymphoid organs (Madej et al., 2015; Madej and Bednarczyk, 2016), and increase the resistance to heat stress (Yan et al., 2019; Jiang et al., 2020; Hu et al., 2022) has been consistently documented. In addition, synbiotics may help to decrease the intestinal and carcass load of various harmful bacteria, including *Campylobacter* (Baffoni et al., 2017), *Clostridium perfringens* (Abd El-Ghany et al., 2010; Shanmugasundaram et al., 2020) and *Salmonella enterica* serovar *Enteriditis* (Markazi et al., 2018; Shanmugasundaram et al., 2019; Sobotik et al., 2021).

Since most of the experiments on synbiotics have been conducted in broilers, less is known about their possible applications in other productive categories, whose different genetic features and farming systems entail different challenges and requirements. For this reason, this study aimed to evaluate the benefits of a multi-species synbiotic product on broiler breeders, by assessing its effects on performance and gut health during the rearing and laying periods.

MATERIALS AND METHODS

Ethical approval

Ethical review and approval were waived for this study since animals were sampled during commercial activities in the farm regulated by national and international laws.

Experimental design

The present field study was conducted in a private broiler breeder farm located in the region of Ioannina, Greece, and covered the first 40 weeks of age of the chickens. A total of 24761 day-old Ross 308 parent stock chicks were supplied from the same hatchery and placed in separate houses on the same farm. In detail, 6200, 6264, and 8937 females were placed in houses A, B, and C, respectively. The synbiotic was administered to houses A and B, while house C acted as a control group. A total of 3360 males were raised in a separate house and were introduced in houses A, B, and C at the age of mating (19 weeks) with a ratio of one male to 10 females.

Management

To ensure flock welfare and achieve high performance, management conditions followed the official guidelines for parent stocks (Aviagen, 2018). Chickens were placed on a floor covered with straw (deep litter system) and were fed *ad libitum* for the first 2 weeks. Restricted daily feeding was observed from the second to the fourth week; then, starting from week 4, the feed was supplied on a skip-a-day regimen. Feed allocation followed the recommendations for breeders, weighing the chickens weekly and adjusting the dose accordingly (Aviagen, 2018). The light period was 20 hours in the first week, 12 hours in the second week, and 8 hours from the third to week 21. From week 21 onwards, the light period was increased from 8 hours up to 14 hours based on average BW and weight uniformity. The temperature was set according to official guidelines, starting at 30°C at the chicks' arrival and decreasing by 1°C every three days

until day 27, then keeping it at 20°C for the rest of the productive cycle. The relative humidity was kept at 60-70% (Aviagen, 2018). Stocking densities were seven female chickens/m^2 and five male chickens/m^2, as indicated by EFSA (2010).

The diet was formulated in accordance with the official genetic line guidelines (Aviagen, 2016), implementing a seven-phase feeding system (starter 1, 0-21 days; starter 2, 22-35 days; grower, 36-105 days; pre-breeder, 106 days to 5% production; breeder 1, 5% production to 245 days; breeder 2, 246-350 days; breeder 3, after 351 days). The exact nutrient specifications are provided in Table 1. Water was provided *ad libitum.*

Chickens were vaccinated at the hatchery against infectious bursal disease (IBD) and Marek's disease (MD). The full vaccination protocol was administered throughout the cycle, including vaccines against infectious bronchitis (IB), Newcastle disease (ND), avian rhinotracheitis (ART), chicken infectious anemia (CIA), infectious avian encephalomyelitis, *Escherichia coli*, salmonellosis, and coccidiosis (Table 2). No antibiotics were administered throughout the considered period.

Table 1. Nutrient composition of the seven-phase feeding system observed to raise the Ross 308 broiler breeders used in the experiment

Diet	**Starter 1 (days 1-21)**		**Starter 2 (days 22-35)**		**Grower (days 36-105)**		**Pre-Breeder (day 106 to 5% production)**		**Breeder 1 (5% production to day 245)**		**Breeder 2 (days 246-350)**		**Breeder 3 (after day 351)**	
Energy	2800 kcal/kg		2800 kcal/kg		2600 kcal/kg		2700 kcal/kg		2800 kcal/kg		2800 kcal/kg		2800 kcal/kg	
Amino acids (%)	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**
Lysine	1.06	0.95	0.74	0.67	0.58	0.52	0.58	0.52	0.67	0.60	0.62	0.56	0.58	0.52
Methionine + Cysteine	0.84	0.74	0.67	0.59	0.59	0.52	0.58	0.51	0.67	0.59	0.65	0.57	0.59	0.54
Methionine	0.51	0.46	0.41	0.37	0.36	0.33	0.35	0.32	0.41	0.37	0.40	0.36	0.36	0.35
Threonine	0.75	0.66	0.60	0.53	0.50	0.44	0.47	0.41	0.55	0.49	0.53	0.47	0.51	0.47
Valine	0.80	0.71	0.70	0.63	0.49	0.44	0.51	0.45	0.63	0.56	0.60	0.53	0.57	0.51
IsoLeucine	0.70	0.62	0.62	0.55	0.45	0.40	0.47	0.41	0.56	0.50	0.54	0.48	0.51	0.45
Arginine	1.17	1.05	0.93	0.83	0.71	0.64	0.74	0.67	0.88	0.79	0.86	0.77	0.80	0.72
Tryptophan	0.19	0.16	0.18	0.15	0.14	0.12	0.15	0.13	0.16	0.14	0.15	0.13	0.14	0.12
Leucine	1.23	1.11	0.93	0.83	0.77	0.69	0.80	0.72	1.04	0.94	1.00	0.90	0.96	0.86
Crude Protein	19.00		17.00		13.00-14-00		14.00		15.00		14.00		13.00	
Minerals (%)														
Calcium	1.00		1.00		0.90		1.20		3.00		3.20		3.40	
Available Phosphorus	0.45		0.45		0.42		0.35		0.35		0.33		0.32	
Sodium	0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23	
Chloride	0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23	
Potassium	0.40-0.90		0.40-0.90		0.40-0.90		0.60-0.90		0.60-0.90		0.60-0.90		0.60-0.90	
Added trace minerals (mg/kg)														
Copper	16								10					
Iodine	1.25								2.00					
Iron	40								50					
Manganese	120								120					
Selenium	0.30								0.30					
Zinc	110								110					
Minimum specifications														
Choline (mg/kg)	1400		1400		1300		1200		1200		1050		1050	
Linoleic acid (%)	1.00		1.00		1.00		1.00		1.25		1.25		1.25	
Added Vitamins/Kg	**Wheat-based feed**				**Maize based feed**				**Wheat-based feed**				**Maize based feed**	
Vitamin A (IU)	11000				10000				12000				11000	
Vitamin D3 (IU)	3500				3500				3500				3500	
Vitamin E (IU)	100				100				100				100	
Vitamin K (mg)	3				3				5				5	
Thiamin (B1) (mg)	3				3				3				3	
Riboflavin (B2) (mg)	6				6				12				12	
Nicotinic Acid (mg)	30				35				50				55	
Pantothenic Acid (mg)	13				15				13				15	
Pyridoxine (B6) (mg)	4				3				5				4	
Biotin (mg)	0.20				0.15				0.30				0.25	
Folic Acid (mg)	1.50				1.50				2.00				2.00	
Vitamin B12 (mg)	0.02				0.02				0.03				0.03	

Table 2. Vaccination protocol administered at the hatchery and throughout the production cycle on the Ross 308 broiler breeders used in the experiment

Age (day)	Vaccine(s)	Disease(s)	Route
day 18 of incubation	Cevac® MD HVT+Rispens	Marek's disease	*In ovo* injection
Hatch day	Cevac® Transmune IBD	Infectious bursal disease virus	Subcutaneous injection
1	Nobilis® IB H120 + Cevac® IBird + Poulvac® E. coli	Infectious bronchitis + colibacillosis	Spray
2	Gallivac® Se + AviPro Salmonella VAC T	Salmonellosis (*Salmonella enteriditis and Typhimurium*)	Water
6	Paracox®	Coccidiosis	Spray/Water
10	Avinew®	Newcastle disease	Spray/Water
18	Nobilis® IB 4/91	Infectious bronchitis	Spray/Water
28	Nobilis® IB Ma5+ Nobilis® ND Clone 30	Infectious bronchitis + Newcastle disease	Spray/Water
35	Nemovac	Avian rhinotracheitis	Spray
50	Gallivac® Se + AviPro Salmonella VAC T	Salmonellosis (*Salmonella Enteriditis* and *Typhimurium*)	Water
55	Avinew®	Newcastle disease	Spray/Water
70	Nemovac	Avian rhinotracheitis	Spray
78	Nobilis® IB Ma5 + Nobilis® ND Clone 30	Infectious bronchitis + Newcastle disease	Spray/Water
88	Nobilis® IB 4/91	Infectious bronchitis	Spray/Water
92	AviPro Thymovac®	Chicken infectious anemia	Water
100	Nobilis® ND Clone 30+ Poulvac® *E. coli*	Newcastle disease + colibacillosis	Spray
107	AviPro AE®	Infectious avian encephalomyelitis	Water
125	Gallimune® 303 + Gumboriffa® + Gallimune® SE+ST + Hiprapox®	Newcastle disease + infectious bronchitis + avian rinotracheitis + infectious bursal disease + salmonellosis (*Salmonella Enteriditis* and *Typhimurium*)+ fowlpox	Intramuscular injection- wing web stab
154	Avinew®	Newcastle disease	Water
224	Nobilis® IB Ma5 + Avinew®	Infectious bronchitis, Newcastle disease	Water

Synbiotic administration

The synbiotic product PoultryStar® sol (BIOMIN GmbH, Getzersdorf, Austria), containing patented probiotic strains plus prebiotic fructooligosaccharides, was applied in houses A and B by drinking water based on a protocol planned with the manufacturer's guidance. In detail, a daily dosage of 20 g/1,000 chickens was supplied for three consecutive days during weeks 1 and 21 (the first administration after males were introduced) and for one day every two weeks during the rest of the cycle of the product.

Sample collection

Ten randomly selected chickens per treatment group were euthanized by cervical dislocation at 15, 25, and 40 weeks of age to collect specimens for histopathological analysis and lesion scoring. About 3 g of caecal content was also collected to evaluate the microbial composition.

Performance parameters

Live BW and mortality were recorded on a weekly and daily basis, respectively, and egg production was expressed on a hen-day basis from the beginning of the production period (23 weeks) up to 40 weeks. Egg fertility and hatchability were recorded as a percentage of total settable eggs throughout the laying period.

Egg quality traits

At week 30, from the beginning of the laying period, 20 eggs per group were randomly collected every two weeks up to week 40 to assess several external and internal egg traits. Individual eggs were weighed to the nearest 0.01 g accuracy with a digital balance, and the egg length and breadth were measured using digital calipers. A shape index was then calculated by dividing the breadth by the length and multiplying by 100. The shell strength was measured using TA.HD plus Texture Analyser (Stable Micro Systems Limited, Godalming, UK). Shell weight

was measured after removing the inner shell membrane and keeping it dry for 24 hours. Shell thickness was evaluated using the Egg Shell Thickness Measure Model 25-5 (B.C. Ames Incorporation, Melrose, Massachusetts) by considering the average of three equidistant points on the equator. The albumen height was measured with the Egg Quality Micrometers S-8400 spherometer (B.C. Ames Incorporation, Melrose, Massachusetts) at 3-4 locations and averaged. The yolk and albumen were weighed to the nearest 0.01 g accuracy on a digital balance. The Haugh unit (HU) was calculated using the formula HU = 100 logs (H+7.57-1.7 W37), where H is the height of the albumen in millimeters and W is the egg weight in grams.

Bacterial enteritis scoring

A macroscopic lesion scoring system was applied to evaluate the chickens' intestinal health in each group at three different time points. Specifically, ten parameters (De Gussem, 2010) were assessed by visual inspection of the intestinal wall during the necropsy. Each parameter was scored 0 when absent and 1, summed and divided by 2.5, resulting in a total score between 0 (normal gastrointestinal tract) and 4 (severe dysbacteriosis) (De Gussem, 2010; Teirlynck et al., 2011).

Histology

Segments of 3 cm were collected from the duodenum, jejunum, ileum, and caecum, keeping the collection sites consistent for each tract. All samples were placed in individually labeled flasks containing 10% neutral buffered formalin, as described by Hoerr (2001). Transversal sections approximately 1 mm thick of each sample were then cut after 48 hours. Sections of 3-5 μm were taken, stained with hematoxylin and eosin, and evaluated. The histopathological and morphometrical evaluation of specimens was performed blindly. The scoring system proposed by Kraieski et al. (2017) was adopted to assess the degree of inflammation in each section. Specifically, the severity of the lesions was graded on a 0-3 scale: 0 corresponded to absent or rare leukocytic infiltration, 1 to leukocytic infiltration up to 5% of a field at x400, 2 to approximately 25% leukocytic infiltration of a field at the same magnification, 3 to leukocytic infiltration in the range of 50%. The morphometry of the intestinal villi and crypts was examined using optical capture and measurement with Image Pro-Plus version 6.0 software (Media Cybernetics, Silver Spring, MD). The selection of the villi for the morphometrical analysis was conducted according to Gava et al. (2015), considering only those that had their bases embedded in the submucosa, without any discontinuity or folds in their length, and with intact epithelium at the tip.

Evaluation of enteric microbiota

High-throughput sequencing was performed on a total of 64 samples, consisting of 10 caecal content from each treatment group. For each sampling point, two meconium samples from the breeders' grandparents (sequencing controls) and two water samples (contamination controls). The analysis was performed on an Illumina MiSeq System (Illumina, San Diego, California) at BioLizard (Ghent, Belgium), LGC genomics (Berlin, Germany) targeting the V3 region of the 16S rRNA gene, and generated 2 x 300 paired-end sequences. Following a preliminary evaluation of the read quality of unmerged sequences with FastQC 0.11.9, the forward reads were trimmed at 195 bp, and the reverse reads at 220, ensuring a minimal Phredscore of 28. The amplicon sequence variants (ASVs) that most accurately describe the data were inferred with DADA2 (Callahan et al., 2016), and then the forward and reverse reads were merged, setting the minimal overlap to 12 bp. After removing chimeric sequences from the dataset, the SILVA 138 reference database (Quast et al., 2013; Yilmaz et al., 2014) was used to classify ASVs as taxons.

Four diversity indexes (Simpson, Shannon, Chao1, and Observed species index) were used to calculate the alpha diversity. Permutational ANOVAs were performed on the euclidean distances between samples for significance testing between groups. Since these tests require an adequate homogeneity of the separate group dispersions, this assumption was first verified with the betadisper function from the vegan R package (Dixon, 2003). To verify the presence of no systematic biases or confounding effects, the Spearman correlation of the treatment effect with other variables (such as age, weight, bacterial enteritis score, histological lesion scores, crypts, villi length, etc.) was run. Differential abundance analysis was then performed with DESeq2 to evaluate the isolated effect of the treatment and the other factors.

Statistical analysis

Data were organized and analyzed in R version 3.3.2 (R Core Team, 2013). For each considered variable, the statistical significance of between-treatment differences was evaluated at each time point using a Student t-test or, if relative assumptions were violated, the non-parametric Mann-Whitney test. Differences between the three houses were evaluated using ANOVA or, in case the relative assumptions were not met, with the Kruskal-Wallis test

followed by post-hoc Mann-Whitney test with Bonferroni correction. Survival analysis was performed using the survival library in R. Kaplan-Meier cumulative survival curves were calculated, and the significance of the difference between treatment groups in the survival curves was assessed using the Log-rank (M-H). The significance level was set to $p < 0.05$. The statistical evaluation of sequencing data was performed independently at BIOLIZARD NV (Ghent, Belgium). For differential abundance analysis, the significance level was set to $p < 0.01$.

RESULTS

Bacterial enteritis and histopathological lesion scores

The BE score measured in the control group was higher than in the treated chickens at every time point, with a statistically significant difference ($p = 0.049$) observed at week 25 (Graph 1). No significant differences were found between houses. As for the histopathological lesion score, lower and statistically significant scores were found in the synbiotic-treated chickens than in control ones at week 25 in the caecum ($p = 0.025$), and at week 40 at caecum ($p = 0.021$) and ileum ($p = 0.002$). Conversely, the control group showed a lower score than treated chickens in the jejunum at week 25 ($p = 0.032$, Graph 2). No significant differences ascribable to the house effect were found at between the two treatment houses at duodenum level at week 15 ($p = 0.42$), week 25 ($p = 0.6$) and week 40 ($p = 0.18$); at jejunum level at week 15 ($p = 0.42$), week 25 ($p = 0.6$) and week 40 ($p = 1$); at ileum level at week 15 ($p = 0.42$), week 25 ($p = 1$) and week 40 ($p = 0.27$); and at caecum level at week 15 ($p = 0.42$), week 25 ($p = 0.27$) and week 40 ($p = 0.42$).

Evaluation of intestinal villi and crypts

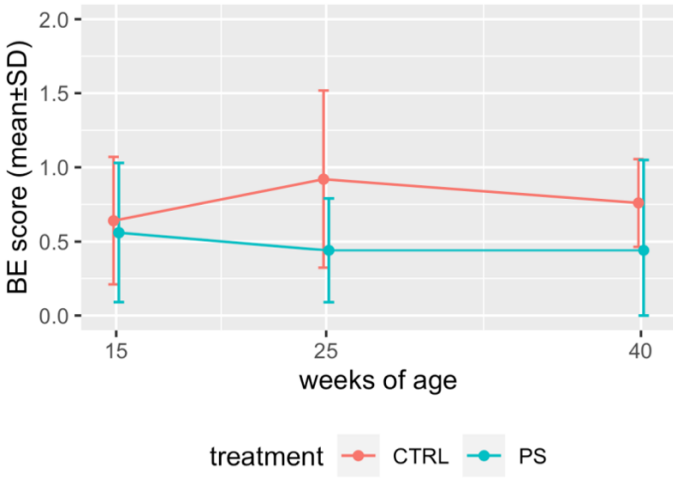
As shown in Graph 3, several differences could be observed between treated and control animals in terms of gut morphometric parameters. Considering only significant differences, synbiotic-treated chickens showed longer villi than control chickens at week 15 in the ileum ($p = 0.004$), at week 25 at the duodenum ($p < 0.0001$), jejunum ($p < 0.0001$), ileum ($p = 0.001$) and caecum ($p < 0.0001$) level, and again at week 40 in all four tracts (all with $p < 0.0001$). Less consistent differences were observed when measuring the crypts, which were significantly deeper in synbiotic-treated than in control chickens in the duodenum at week 25 ($p < 0.0001$) and in the jejunum tract at week 15 ($p < 0.0001$) and week 40 ($p = 0.0004$), but less deep in the caecum at week 25 ($p = 0.002$). The house effect on villi length was significant in the duodenum at week 15 ($p = 0.005$), in the jejunum at week 25 ($p < 0.0001$) and week 40 ($p = 0.009$), in the ileum at week 40 ($p = 0.006$) and in the caeca at week 25 ($p = 0.007$). In terms of crypt length, houses A and B differed significantly at week 25 at the duodenum ($p < 0.0001$) and jejunum level ($p = 0.006$, Graph 4).

Performance

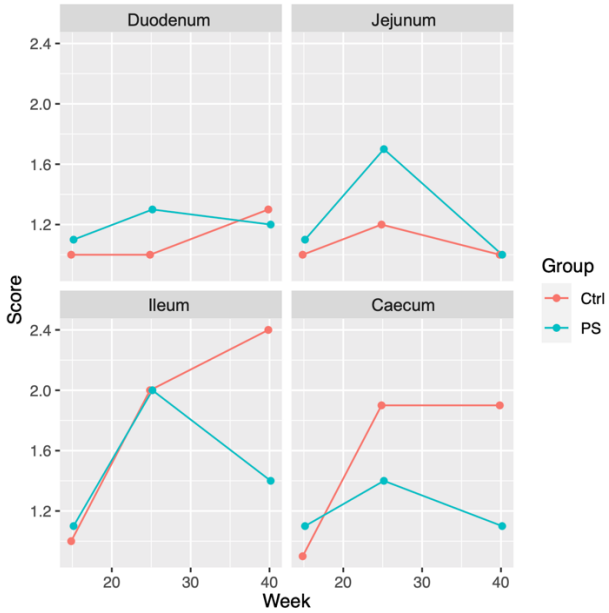
There was a significant between synbiotic-treated chickens and the control group in terms of live BW, (Graph 5, $p = 0.05$). However, the house effect seemed far more relevant in determining the observed differences ($p < 0.0001$), as house C (control) performed better than house B but worse than house A. In particular, the biggest difference was observed in the BW of males, which was remarkably higher for house A ($p < 0.0001$ when compared to both houses B and C). On the other hand, the BW of producing hens was less heterogeneous, and better performance was observed in house C than in the treated houses ($p < 0.001$ for both comparisons, Graph 5b). A significant difference in terms of survivability throughout the production period (23-40 weeks) was observed between the treated and control groups ($p < 0.001$) (Graph 6a). Significant differences were also observed when considering the three houses separately ($p < 0.001$), with both treatment houses scoring better than the control (Graph 6b). No significant differences were found in terms of egg fertility and hatchability, neither between synbiotic-treated and control chickens ($p = 0.12$ for egg fertility, $p = 0.67$ for hatchability) nor between treated houses ($p = 0.1$ for egg fertility, $p = 0.47$ for hatchability).

Egg quality traits

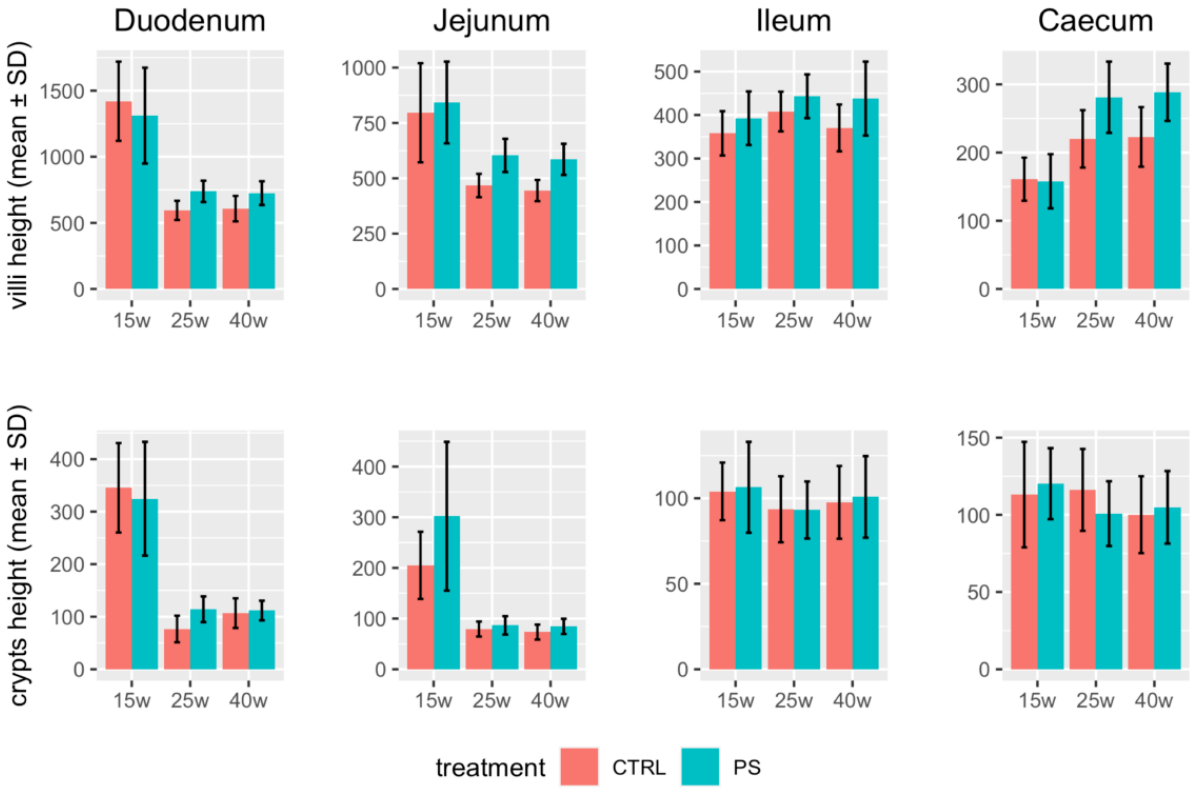
There were no significant differences in terms of eggshell strength, shell thickness, and shape index, but some were found at limited time points in egg weight, shell weight, and combined albumen and yolk weight between treatments and, more limitedly, between houses. In particular, the egg weight was higher in synbiotic-treated chickens than in control ones at week 30 ($p = 0.009$) but lower at week 40 ($p = 0.032$). Shell weight was higher in synbiotic-treated chickens than in control ones at week 30 ($p = 0.018$). The combined weight of yolk and albumen was higher in control chickens than in synbiotic-treated ones at week 40 ($p = 0.026$). Overall, no clear trends that could be ascribable to the synbiotic treatment were identified (Graph 7).



Graph 1. Bacterial enteritis score measured in synbiotic-treated and control broiler breeders



Graph 2. Histopathological lesion scores measured in different intestinal tracts in synbiotic-treated and control broiler breeders



Graph 3. Gut morphometric parameters measured in different enteric tracts in synbiotic-treated and control chickens

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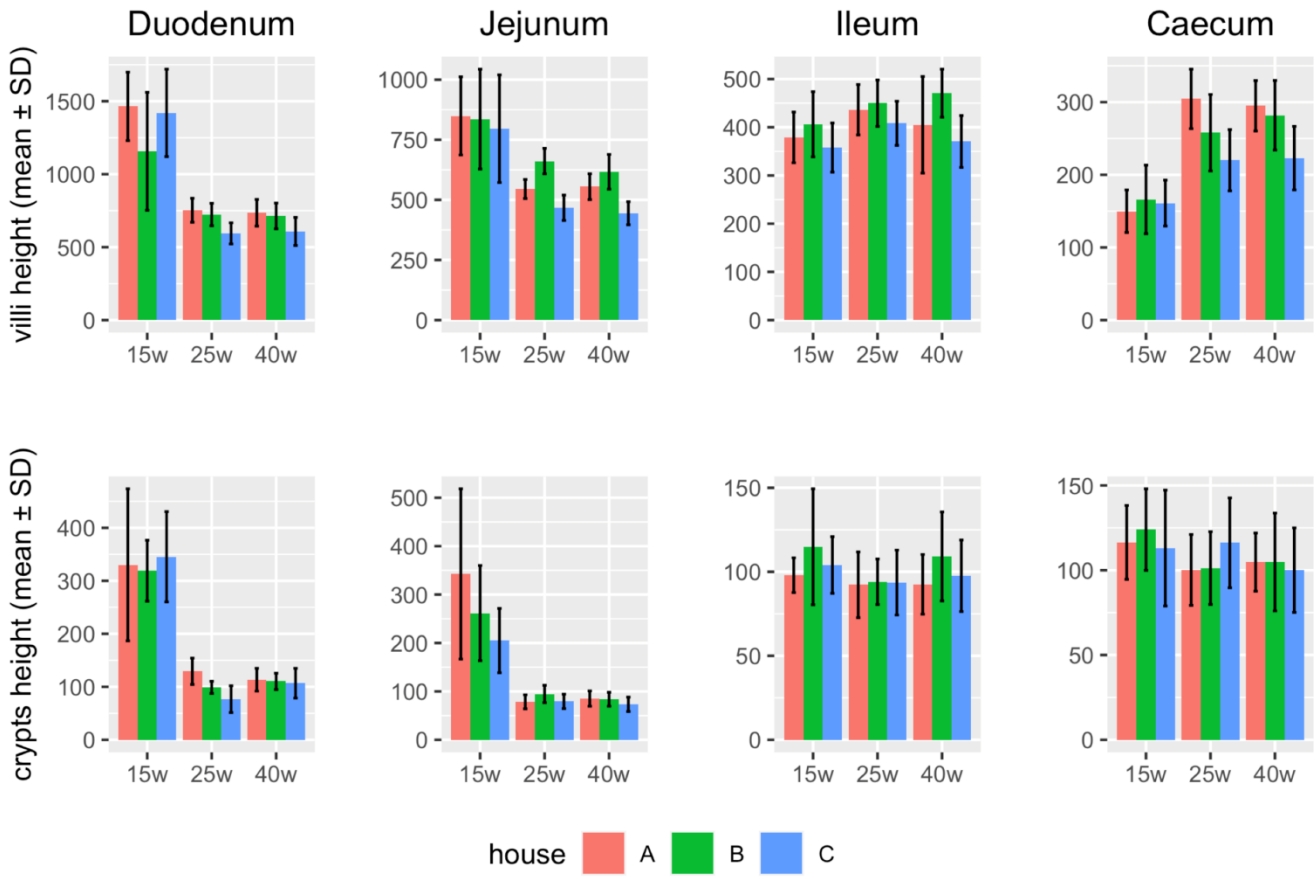
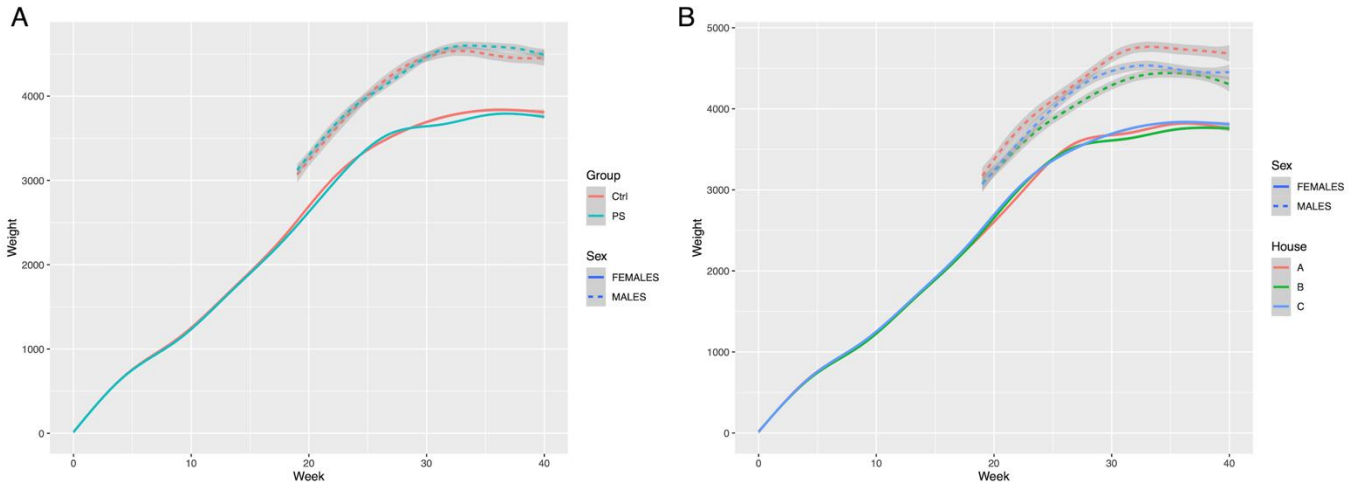
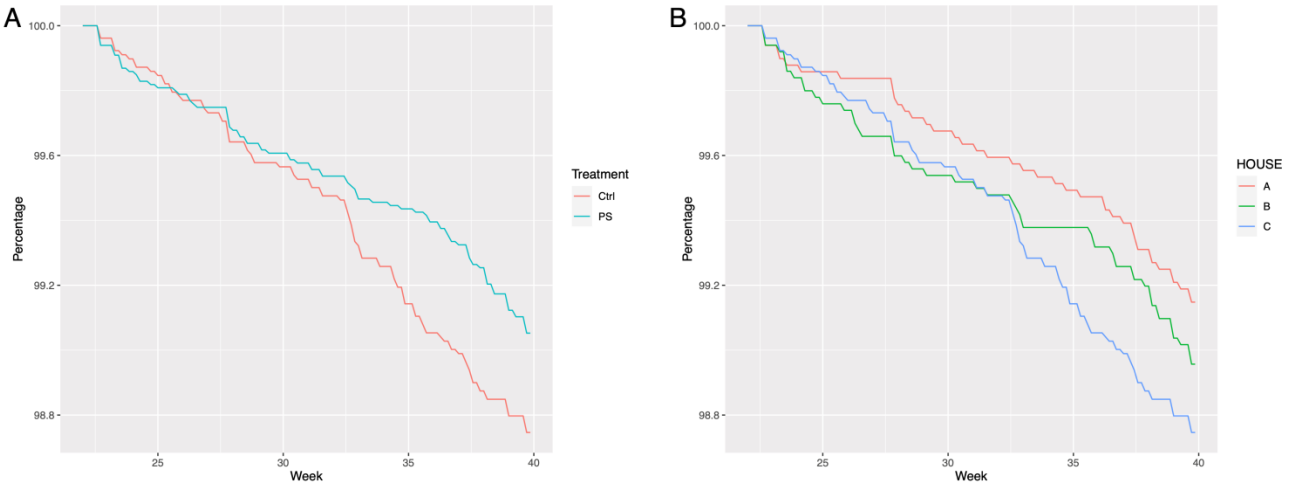


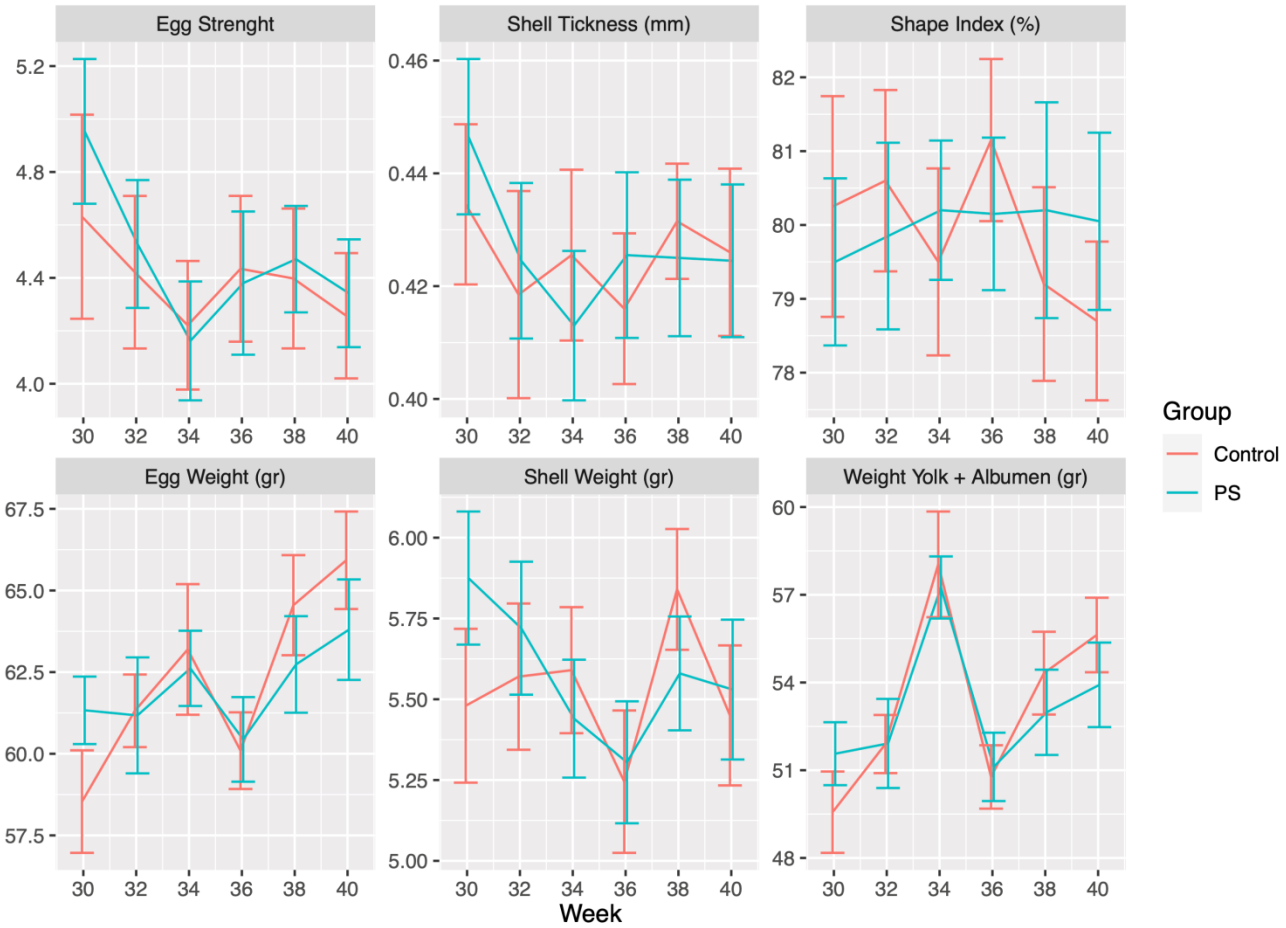
Graph 4. Gut morphometric parameters measured at 15, 25, and 40 weeks of age in different enteric tracts of the broiler breeders raised in the three houses. The synbiotic was administered in houses A and B, while house C acted as the control group



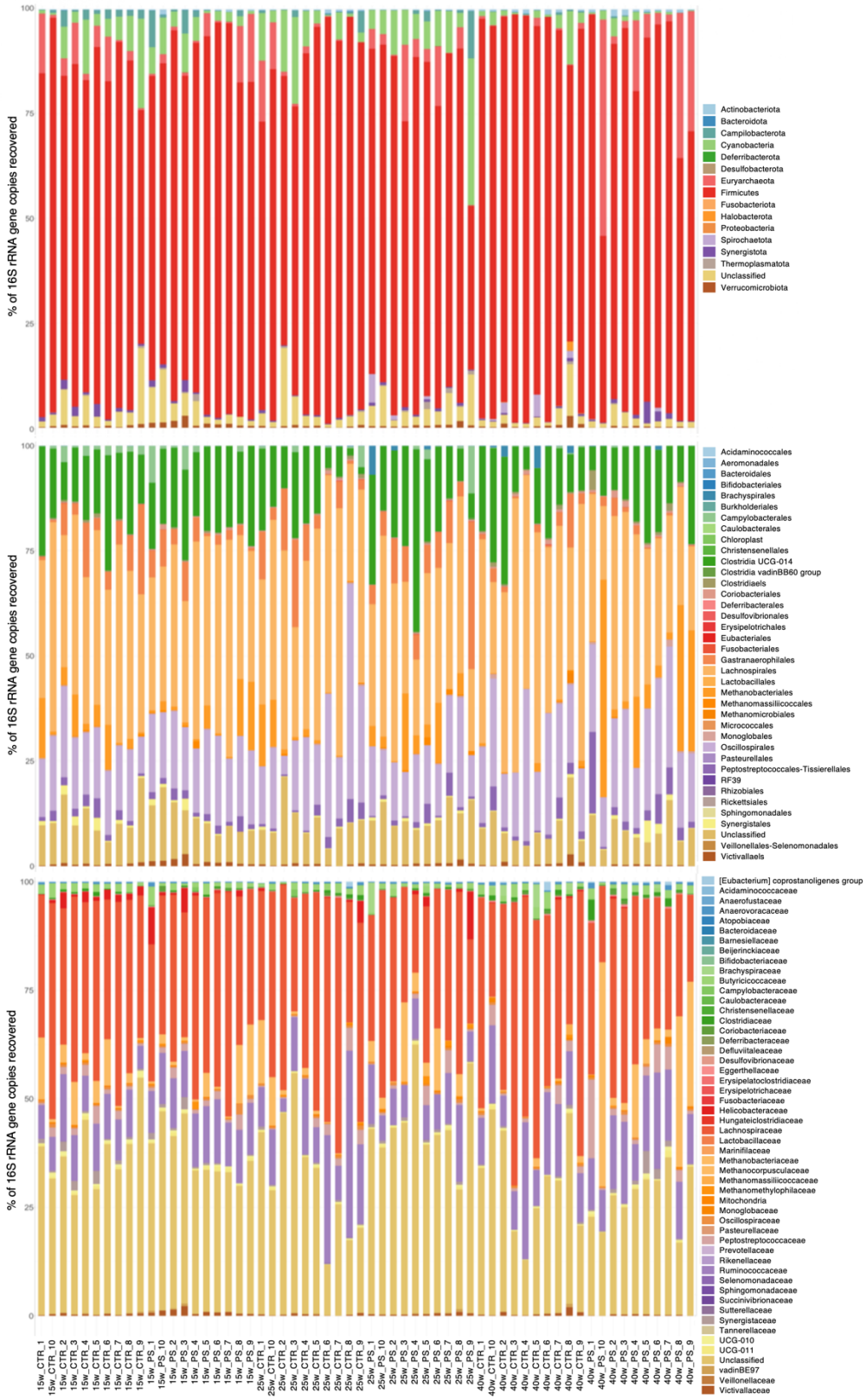
Graph 5. Growth curves comparison between synbiotic-treated and control broiler breeders (a) and between the three houses (b). The synbiotic was administered in houses A and B, while house C acted as the control group



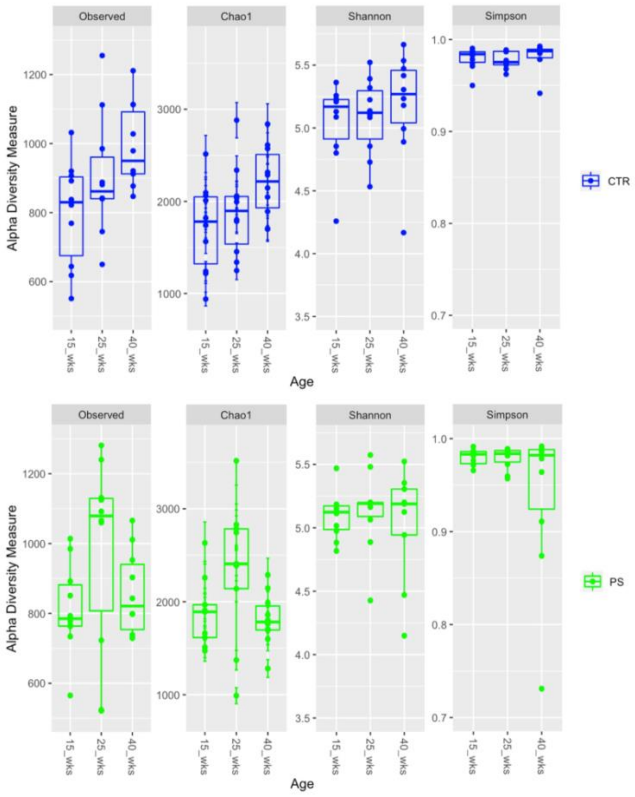
Graph 6. Comparison of survivability rates during the production period (23-40 weeks) between synbiotic-treated and control female broiler breeders (a) and between the three houses (b). The synbiotic was administered in houses A and B, while house C acted as the control group.



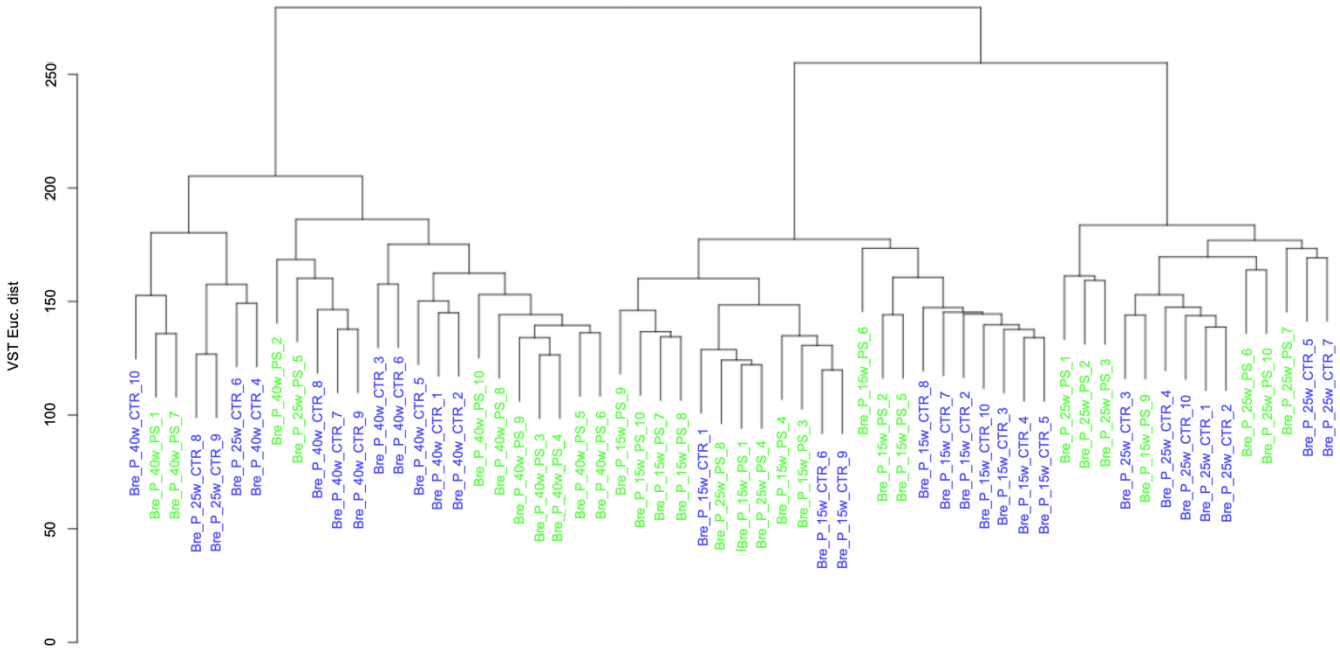
Graph 7. Comparison of egg traits between synbiotic-treated and control broiler breeder chickens



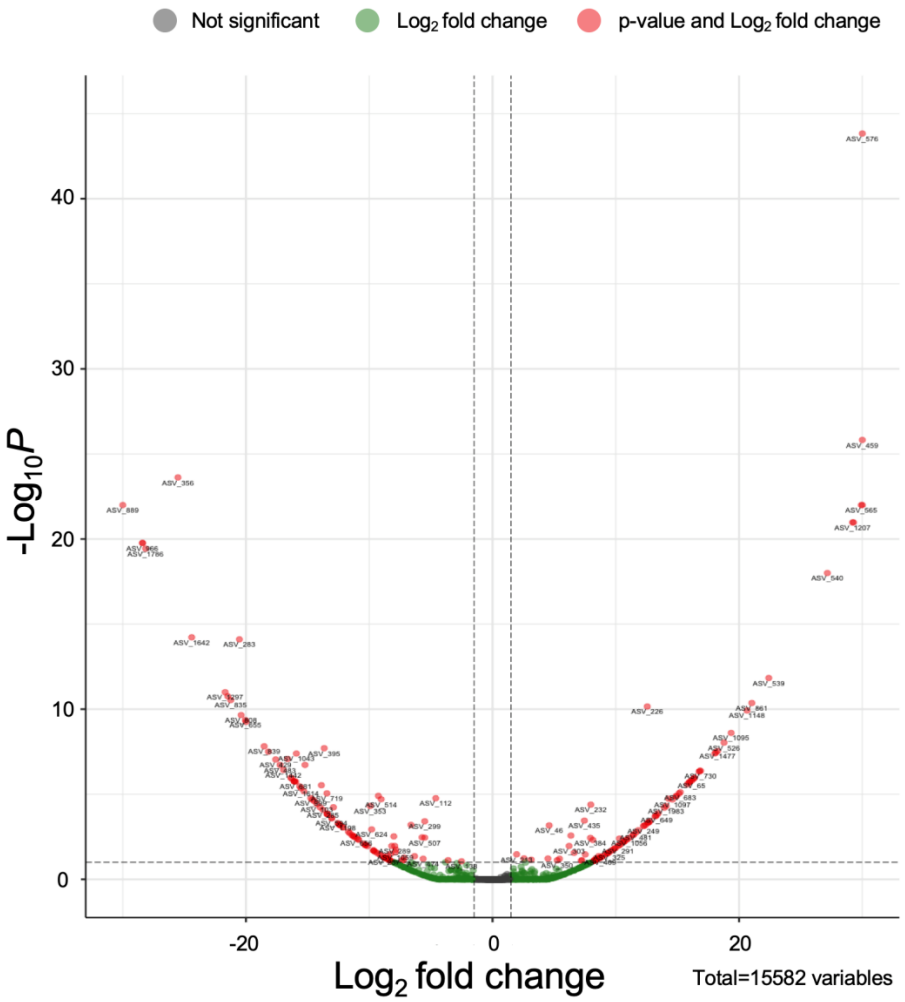
Graph 8. Relative microbial composition of caecal content of synbiotic-treated and control broiler breeder chickens, shown at Phylum (top), Order (centre) and Family (bottom) level.



Graph 9. Alpha-diversity indexes measured in synbiotic-treated (PS) and control (CTR) broiler breeder chickens and divided per age group.



Graph 10. Dendrogram of the broiler breeder caecum samples, clustered on the Euclidean distance between their count data. Sample names are colored green for synbiotic-treated chickens and blue for control chickens. The age at sampling (15, 25 and 40 weeks) is indicated in the code of each sample.



Graph 11. Volcano plot showing the differential abundance of amplicon sequence variants in the caecal microbiota of broiler breeders due to the synbiotic treatment effect. The statistical significance value was set to $p < 0.01$ (horizontal line), while, to be considered biologically significant, the effect size expressed in terms of Fold Change (FC) should have had an absolute value of 3 (vertical lines at $\log_2$ FC = 1.5).

Evaluation of enteric microbiota

According to sequencing results, the overall diversity in the caecum samples was rather high, with a total of 15582 different ASVs. The relative microbial abundance of each caecal content is shown in Graph 8.

According to the measured diversity indexes, the richness of different bacterial species was rather high in most of the samples and generally increased between weeks 15 and 25. A less evident trend was observed from week 25 to 40, when the bacterial diversity in the synbiotic-treated chickens was even shown to decrease (Graph 9).

Hierarchical clustering on euclidean distance showed that samples tended to cluster based on treatment and age, with clear segregation between 15-week-old and 40-week-old chickens and only a slight overlap of 25-week-old chickens with both groups (Graph 10).

A significant treatment effect was found by comparing the microbial composition of samples from synbiotic-treated and control chickens ($p = 0.025$). When the comparisons were between same-age chickens, the treatment effect was significant at week 15 ($p < 0.001$) and week 40 ($p = 0.03$), but not significant at week 25 ($p = 0.064$). The age effect was confirmed significant by comparing samples taken at different ages, both among treated and control chickens ($p < 0.001$ in both cases). Since synbiotic-treated chickens were reared in two separate houses, the possible house effect was also investigated but was found to be non-significant ($p = 0.083$). Intercorrelation analysis revealed no significant

Spearman correlation of any variables to the treatment, indicating a proper experimental setup. When isolating the treatment effect, significant differences were detected in the abundance of 119 out of a total of 15582 ASVs (after Benjamini-Hochberg multiple testing correction, Graph 11). In particular, 45 ASVs were more abundant in the treated breeders, while 74 were less abundant. Among others, the treatment effect seems to have affected the relative abundance of *Gastranaerophilales*, *Helicobacter*, *Ruminococcaceae*, *Lachnospiraceae*, and *Clostridia* (Table 3).

Table 3. Top 10 differentially abundant amplicon sequence variants for the treatment effect ranked on the adjusted p-value. The direction of differential abundance can be inferred from the sign of the Log2 Fold Change

Amplicon sequence variant	Log2 fold change	Standard error	Adjusted p-value	Lowest resolved taxon
ASV_576	30.000000	2.057181	1.4620e-44	Gastranaerophilales
ASV_459	30.000000	2.643062	1.4979e-26	*Helicobacter*
ASV_356	-25.517909	2.349552	2.3997e-24	*Ruminococcaceae*
ASV_565	29.919828	2.863515	1.0061e-22	*Lachnospiraceae*
ASV_797	30.000000	2.870802	1.0061e-22	Bacteria
ASV_889	-29.994918	2.864174	1.0061e-22	Gastranaerophilales
ASV_1207	29.208433	2.864828	1.0554e-21	Clostridia UCG-014
ASV_1822	29.286145	2.872488	1.0554e-21	Clostridia UCG-014
ASV_966	-28.400792	2.867760	1.7165e-20	Clostridia UCG-014
ASV_1298	-28.383064	2.867419	1.7165e-20	Clostridia

DISCUSSION

The present results comprehensively depict the effects of the considered synbiotic product on the performance and gut health of broiler breeders. Following a protocol devised with the manufacturer's guidance, PoultryStar® sol was administered for three consecutive days of weeks 1 and 21, as recommended for newly hatched poultry and around stressful periods and changes, such as the introduction of males. An intermittent schedule was observed throughout the rest of the cycle, which is recommended to support gut eubiosis continuously.

Regarding the obtained results, it is useful to compare them to those obtained in previous trials of other synbiotics, bearing in mind that the outcomes may differ depending on each product's composition, dosage, administration route, and timing, along with environmental and host-related factors.

The effect of PoultryStar® sol administration on BW gain appeared limited, and the observed heterogeneity between the different groups seemed more easily ascribable to the house effect. Several synbiotics, mostly tested on broilers, were shown to increase BW gain and feed conversion ratio (Mohammed et al., 2018; Kridtayopas et al., 2019; Abdel-Wareth et al., 2019), while others had no impact on BW or feed conversion ratio (Chang et al., 2019; Dankowiakowska et al., 2019; Shanmugasundaram et al., 2020). Ultimately, it should also be considered that breeders' feeding programs are targeted at maintaining high weight uniformity and keeping close to BW targets, rather than maximizing growth and feed efficiency (Aviagen, 2018). Any overperformance compared to target BW during both rearing and production periods, may be compensated with feed restrictions (EFSA, 2010), thus masking any potential increase in feed efficiency related to synbiotic administration.

Egg production and quality were also evaluated, as several synbiotics were shown to improve them. Luoma et al. (2017) found that administering a multi-species synbiotic increased egg production between 19 and 28 weeks of age, even after the chickens were challenged with *Salmonella enterica* serovar Enteritidis. Similar results were obtained by Radu-Rusu et al. (2010), Abdel-Wareth (2016), and Tang et al. (2017), who also reported a positive effect on egg quality, resulting in heavier, larger eggs with thicker shells. According to Buyarov and Metasova (2019), synbiotic-fed broiler parent stocks also showed an increase in egg production and hatchability. On the other hand, other tested probiotics and synbiotics had limited or no effect on laying performance (Tang et al., 2015; Liu et al., 2019; Sjofjan et al., 2021). In the present study, no significant differences were found in terms of egg fertility, hatchability, and morphology, except for specific sampling points in terms of egg weight, shell weight, and combined albumen and yolk weight. Based on

these findings, the tested synbiotic did not seem to affect egg production.

A significant treatment effect was found in terms of survivability during the laying period, with both treated groups exhibiting lower mortality than the control one. The decision to focus on the production phase was taken because mortality rates in the rearing phase may be easily altered by culling procedures, which are often due to factors unrelated to the breeders' health, such as chickens not meeting selection criteria or sexing errors (EFSA, 2010). The observed differences suggest that PoultryStar® sol can effectively reduce mortality in field conditions, as already reported for other synbiotics (Awad et al., 2009; Abdel-Wareth et al., 2019; Rodrigues et al., 2020).

Although the ultimate goal of synbiotic administration is to have healthier and, thus, more productive chickens, the evaluation of performance parameters only offers a partial and indirect assessment of their effect on gut health. Ringenier et al. (2021) noted that a healthier intestinal tract does not always correspond to an increase in production parameters, as birds can cope with a certain degree of gut lesions before their performance is affected. For this reason, gut health scores and intestinal morphometry were also considered to assess the effect of PoultryStar® sol in preventing any unfavorable state of inflammation or dysbacteriosis which could negatively alter the integrity of the intestinal mucosa and thus its absorption and immune functions (Willing and Van Kessel, 2009; Teirlynck et al., 2011).

The BE score was lower in treated chickens than in control ones at all time points, with a statistically significant difference at 25 weeks of age. The histopathological lesion score was also significantly lower in the treated groups in the caecum (at 25 and 40 weeks) and ileum (at 40 weeks), while the control group scored better only at a single point at the jejunum level. According to these results, synbiotic-treated chickens exhibited better intestinal health even in the absence of a challenge. This conclusion is supported by the evaluation of gut morphometric parameters, which showed that synbiotic-treated chickens had longer villi consistently along all intestinal tracts from 25 weeks of age onwards. Synbiotic trials often report an increase in villus height in different intestinal tracts, indicating a larger surface for nutrient absorption (Samanya and Yamauchi, 2002) throughout different intestinal tracts (Kridtayopas et al., 2019; Villagrán-de la Mora et al., 2019; Jiang et al., 2020). The effect of PoultryStar® sol on crypts, whose depth is related to the mucosal proliferative activity (Prakatur et al., 2019), appeared less evident and consistent, with deeper crypts being reported in the jejunum and duodenum, while caecal crypts were less deep at 25 weeks of age. Similar findings are reported in previous studies, in which different synbiotic formulations were shown to increase (Villagrán-de la Mora et al., 2019), decrease (Sobolewska et al., 2017), or have no effects (Awad et al., 2009, Sobotik et al., 2021) on crypts depth. It should be noted that the interpretation of the obtained data was complicated by the fact that the two treated houses also exhibited significant differences in villi and crypts length. Nonetheless, the existence of an actual beneficial effect of the synbiotic treatment on intestinal morphology is supported by the overall agreement between the two treated houses compared to the control one, and by the general increase seen in the ratio between villi and crypts length.

The use of high-throughput sequencing provided useful insights into the composition of the caecal bacterial population. However, exactly defining a healthy intestinal microbiota is not an easy task, as it is influenced by a multitude of environmental and host-related factors, such as litter, housing, climate and the chickens' age, sex and breed (Kers et al., 2018). The overall bacterial diversity was rather high and was shown to increase with age, in agreement with previous studies (Videnska et al., 2014; Ocejo et al., 2019). A highly diverse bacterial community is indicative of good intestinal health, while a reduced heterogeneity could signal intestinal disease states (Ocejo et al., 2019; Madlala et al., 2021). The observed caecum composition was in agreement with what was expected in poultry, exhibiting a clear predominance of *Firmicutes*, and, in particular, of families belonging to the class *Clostridia*, such as *Lachnospiraceae*, *Methanobacteriaceae*, and *Ruminococcaceae* (Clavijo and Florèz, 2018; Such et al., 2021). *Firmicutes* are associated with butyrate production, while *Bacteroidetes*, which represent a small fraction of the caecal microbiota, are involved in the production of propionate. Their ratio is commonly accepted as an indicator of the efficiency of energy harvesting in both humans and animals (Zhu et al., 2019). Videnska et al. (2014) studied the development of the caecal microbiota in laying hens over the entire production cycle. They reported that the relative abundance of *Bacteroidetes* increased between the second and the sixth month while *Firmicutes* were predominant during the first month of age, leading to an even ratio between the two phyla in adult hens. Several studies also reported *Firmicutes* to be predominant in broiler chickens and young hens (Bjerrum et al., 2006; Nordentoft et al., 2011; Videnska et al., 2013), while members of

Bacteroidetes seem more abundant in older chickens (Callaway et al., 2009). While this shift has not been observed in the present study, with *Firmicutes* being by far the predominant phyla even at 40 weeks of age, it should be considered that the F/B ratio is heavily determined by the administered feed (Nordentoft et al., 2011) and that it has never been investigated before in broiler breeders, thus preventing comparisons with chickens sharing the same genetic features and producing conditions.

The treatment effect on bacterial composition was confirmed to be statistically significant and led to a differential abundance of 119 ASVs. Among the most impacted were members of the families *Lachnospiraceae* and of the genus *Helicobacter*, which were overrepresented in treated chickens, and of *Ruminococcaceae*, which in turn were underrepresented. More puzzlingly, members of *Gastranaerophilales* and *Clostridia* were found among both the most over and underrepresented ASVs in treated chickens. All these bacteria are common inhabitants of the caecal microbiome (Aruwa et al., 2021; Gilroy et al., 2021; Xiao et al., 2021), and their abundance was already proven to be modulated by several nutraceuticals. Díaz Carrasco et al. (2018) found that tannins administration increased the relative abundance of *Helicobacter* and, more importantly, of members of both *Lachnospiraceae* and *Ruminococcaceae* (and decreased other members of the two families), possibly shifting the short-chain fatty acids caecal profile towards butyrate production. Li et al. (2020) reported that the supplementation of fermented soybean meal in broilers led to an increased abundance of *Gastranaerophilales*, which in turn was positively correlated to an improved average daily gain and serum immunity.

Previous studies relying on high-throughput sequencing already investigated the effect of synbiotics with different compositions on chickens' intestinal microbiota, but, to the authors' knowledge, this is the first time this technique is carried out in broiler breeders, not allowing a comparison with chickens with similar genetic traits and raised under the same production system. Pineda-Quiroga et al. (2019) found that treating laying hens with a synbiotic product based on dry whey powder and *Pediococcus acidilactici* increased the caecal abundance of *Actinobacteria*, *Olsenella* spp., and *Lactobacillus crispatus*, among others. The double administration of a multi-species synbiotic, both by spray at the hatchery and in the feed throughout the broiler cycle, caused an increased abundance of *Actinobacteria* and *Lactobacillus* spp. as well, along with several members of *Clostridia*, and also led to a higher *Firmicutes* to *Bacteroidetes* ratio (Brugaletta et al., 2020). Another trial conducted in broiler chickens found that a synbiotic containing *Bacillus subtilis*, yeast, and inulin did not affect the caecal microbiota (Such et al., 2021). The diversity in the results obtained by these studies can be easily justified by the many variables at play (experimental design, synbiotic composition and dosage, productive type, breed, age at sampling, feed, and rearing conditions) and by the inherent complexity of the caecal ecosystem, which hosts the largest (and partially unculturable) bacterial population out of all intestinal tracts (Aruwa et al., 2021). On the other hand, this adds value to the herein reported data, which are among the first to provide a longitudinal perspective on the enteric microbiome of broiler breeders.

CONCLUSION

Based on the reported results, the synbiotic product PoultryStar® sol appears fully applicable to broiler breeders through intermittent drinking water administration. Histopathological and morphometrical findings support its beneficial effect on gut health, and higher survivability was also observed in treated chickens during the production phase. In addition, the synbiotic treatment had a modulating effect on several bacterial populations hosted in the caeca, whose actual impact will require further investigations to be fully elucidated.

DECLARATIONS

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Authors' contribution

Zoi Prentza contributed to the conceptualization, investigation, data curation, writing, review and editing of the manuscript. Francesco Castellone participated in investigation activities. Matteo Legnardi contributed to data analysis and visualization, writing, review and editing processes. Birgit Antlinger was involved in the conceptualization of the study. Maia Segura-Wang participated in investigation activities. Giorgos Kefalas was involved in the conceptualization process and in resource provision. Paschalis Fortomaris, Angeliki Argyriadou, Nikolaos Papaioannou, and Ioanna Stylianaki participated to data analysis and visualization. Giovanni Franzo participated to data curation, analysis, and visualization. Mattia Cecchinato and Vasileios G. Papatsiros supervised the project. Kostantinos Koutoulis was responsible for the conceptualization, resource provision, supervision, and project administration. All

authors checked and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The funders were not involved in the study design, data collection, and analysis, nor in the writing of the manuscript.

Ethical considerations

All relevant ethical issues have been checked by all the authors.

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