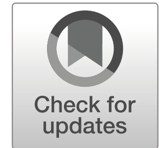
Applied Microbiology and Biotechnology (2020) 104:319–334
https://doi.org/10.1007/s00253-019-10220-7

APPLIED MICROBIAL AND CELL PHYSIOLOGY



Short-term feeding of probiotics and synbiotics modulates caecal microbiota during *Salmonella* Typhimurium infection but does not reduce shedding and invasion in chickens

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Received: 4 September 2019 / Revised: 7 October 2019 / Accepted: 23 October 2019 / Published online: 22 November 2019
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Abstract

Positive modulation of gut microbiota in laying chickens may offer a strategy for reduction of *Salmonella* Typhimurium shedding and production of safer poultry products. In the current study, the caecal luminal microbiota of laying chicks was studied using 16S rRNA amplicon sequencing on DNA obtained from the chicks that were offered supplementation with commercial probiotics, synbiotics and/or *Salmonella* Typhimurium challenge. The load of *Salmonella* Typhimurium in various organs was quantified. Irrespective of the probiotics and synbiotics supplementation and *Salmonella* Typhimurium challenge, caecal microbiota was dominated by 22 distinct bacterial genera and 14 families that clustered into *Actinobacteria*, *Proteobacteria* and *Firmicutes* at phylum level. Taken together, probiotics and synbiotics supplementation increased (false discovery rate; FDR < 0.05) the abundance of *Ruminococcus*, *Trabulsiella*, *Bifidobacterium*, *Holdemania* and *Oscillospira*, indicating their role in maintaining gut health through lowering luminal pH and digestion of complex polysaccharides. *Salmonella* Typhimurium challenge decreased the abundance of *Trabulsiella*, *Oscillospira*, *Holdemania*, *Coprococcus*, *Bifidobacterium* and *Lactobacillus* and increased *Klebsiella* and *Escherichia*, indicating its role in caecal dysbiosis. Although probiotics and synbiotics supplementation positively modulated the caecal microbiota, they were not effective in significantly ($P > 0.05$) reducing *Salmonella* Typhimurium load in caecal tissue and invasion into vital organs such as liver and spleen. The early colonisation of laying chick caeca by probiotics and synbiotics had the potential to positively influence luminal microbiota; however, the microbial abundance and diversity were not sufficient to significantly reduce the shedding of *Salmonella* Typhimurium in faeces or invasion into internal organs during this study.

Keywords 16S rRNA amplicon sequencing · Gut microbiota · *Salmonella* Typhimurium pathogenesis · Laying chicks · Food safety · Microbial abundance and diversity

Introduction

Caecal microbiota in chickens is linked with host health and productive traits that reflect its importance in colonisation resistance to zoonotic pathogens (Shini et al. 2013). Positively influencing the host gut microbiota helps in digestion and metabolism (Stanley et al. 2012), regulation of intestinal angiogenesis (Stappenbeck et al. 2002), development and regulation of host immune system (Hooper et al. 2001) and even in brain function (Benakis et al. 2016). Microbial communities are influenced by tissue type, flock age, disease and rearing conditions (Cui et al. 2017; Luoma et al. 2017; Ngunjiri et al. 2019). Studies suggested that the host genotype can exert a strong influence on gut microbiota composition (Goodrich et al. 2016) and, therefore, the microbiome of egg-type birds is not the same as that of broilers (Ocejo et al. 2019). In layer chicks, the lowest complexity of caecal microbiota is around day 1 of hatch, where it usually consists of five different species (Crhanova et al. 2011). The microbial diversity slowly increases with bird age to 14 species on day 3 and approximately 42 species around day 19 of hatch (Crhanova et al. 2011). After 2 weeks post-hatch, the *Ruminococcus* and *Firmicutes* increase to a greater extent than the *Enterobacteriaceae* (Ballou et al.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-019-10220-7) contains supplementary material, which is available to authorized users.

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2016). Newly hatched chicks are prone to colonisation by pathogenic microorganisms present in the rearing environment. Higher microbial diversity is commonly associated with healthy host conditions, while reduced microbial diversity affects the intestinal health negatively (Sommer et al. 2017).

To modulate the gut microbiota composition in chickens, diets are often supplemented with pre- and probiotics. Prebiotics are non-living fibrous feed additives (non-digestible oligosaccharides) that promote the growth and multiplication of the indigenous gut microbiota. Therefore, prebiotics serve as feed for beneficial indigenous gut bacteria. The proposed mechanisms of action of prebiotics include the production of antimicrobial substances (Chen et al. 2007), modulation of host immune system (Babu et al. 2012) and improving gut morphology (Pourabedin et al. 2014). In contrast to prebiotics, probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Fuller 1989). The protective effects of probiotics in the gut could be due to the production of organic acids (lactic acid) and adhesion inhibitors, and the secretion of antimicrobial substances such as hydrogen peroxide and bacteriocins (Spinler et al. 2008). Probiotics can also secrete enzymes that hydrolyse bacterial toxins and modify toxin receptors (Buts et al. 1994).

In laying hens, diets supplemented with different strains of probiotics have significantly improved gut microbial balance, blood and yolk cholesterol levels, egg production and overall egg quality. For example, *Lactobacillus* improved the equilibrium of gut microbiota by increasing the population of *Bifidobacteria* and decreased potentially harmful bacteria (Forte et al. 2016). *Lactobacillus* and *Bacillus* improved overall birds' performance, caecal microbiota and gut morphology (Forte et al. 2016). *Lactobacillus salivarius* and *Bacillus subtilis* improved egg production, serum low-density cholesterol and serum antibody level against avian influenza virus (Zhang et al. 2012). *Bacillus licheniformis* and *B. subtilis* improved egg production, damaged egg ratio, egg yolk cholesterol and serum cholesterol (Kurtoglu et al. 2004). *Pediococcus acidilactici* positively influenced overall egg quality, feed efficiency and yolk fatty acid composition and yolk content (Mikulski et al. 2012). *Enterococcus faecium* improved overall egg quality, gut microbiota, serum cholesterol level, nutrient digestibility and excreta ammonia emission (Park et al. 2016; Zhang and Kim 2013). Apart from competitive exclusion theory, probiotics may offer protection against pathogens by modulating host immune response. For example, chickens infected with *Salmonella* Typhimurium resulted in reduced production of IFN-γ when fed with *Lactobacillus*, *Bifidobacterium* and *Streptococcus*-based probiotic (Haghighi et al. 2008). Similarly, pre-treatment of human intestinal epithelial cells with *Bifidobacterium or Lactobacillus* resulted in the inhibition of *Salmonella*-induced *IL-8* expression (O'Hara et al. 2006). In laying hens, prebiotics supplementation has been shown to influence gut health and birds performance positively. For example, xylo-oligosaccharides improved intestinal epithelial morphology, caecal *Bifidobacterial* population, caecal butyrate level, plasma immunoglobulins concentrations and plasma vitamin D3 level (Ding et al. 2017). Isomalto-oligosaccharide improved overall egg quality, egg production, feed intake and serum cholesterol level (Tang et al. 2017). Mannan-oligosaccharides positively influenced egg production, egg weight, liver antioxidant status and feed conversion ratio (Bozkurt et al. 2016). Fructooligosaccharides reduced *Salmonella* Enteritidis colonisation in the liver and ovary (Donalson et al. 2008). Inulin reduced yolk cholesterol concentration, caecal pH and coliform bacteria count (Shang et al. 2010).

In healthy adult chickens, *Salmonella* infection generally does not lead to the development of clinical signs (Barrow and Lovell 1991), while in young chicks, it can cause morbidity and mortality (Williams Smith and Tucker 1980). However, *Salmonella* infection potentially changes gut microbial communities dominated by *Enterobacteriaceae* (Liu et al. 2018) with more visible effects in younger chicks (Juricova et al. 2013). In 1-week-old layer chicks, *Salmonella* Enteritidis challenge altered the caecal microbial communities (Mon et al. 2015). The effects of *Salmonella* Enteritis on changes in gut microbiota were greater in day-old chicks compared with 4 and 16-day-old Isa-Brown chicks (Juricova et al. 2013). Pre- and pro-biotics are effective in clearing *Salmonella* from the chicken gut through modification of the gut microbiome (Azcarate-Peril et al. 2018; Bratburd et al. 2018) and host immune system modulation (Chang et al. 2019; Haghighi et al. 2008). *Lactobacillus* has been shown to reduce *Salmonella* Enteritidis load in chickens caeca significantly (Penha Filho et al. 2015). In a mouse colitis model, probiotic *Escherichia coli* Nissle 1917 was effective in reducing *Salmonella* Typhimurium colonisation (Deriu et al. 2013). Multiple strains based probiotic was effective in reducing the shedding level of *Salmonella* Typhimurium in pigs (Casey et al. 2007). A significant interaction of pre- and pro-biotics on host immune response against *Salmonella* Typhimurium has been observed in pigs (Naqid et al. 2015). In humans, gastroenteritis caused by *Salmonella* Typhimurium is often traced back to contaminated poultry produce (Fearnley et al. 2011); hence, the poultry industry is under constant pressure to contain this pathogen at farm level. Based on the intended use of probiotics and synbiotics for controlling *Salmonella* in chickens, we hypothesised that, if used in the first week of the chick's hatch, commercial probiotics and synbiotics can provide colonisation resistance through competitive exclusion against *Salmonella* Typhimurium in the caeca at an early age. To test this hypothesis, we used next-generation sequencing targeting hypervariable regions within microbial 16S rRNA genes to compare the caecal luminal microbiota of layer

chicks exposed to short-term probiotics and synbiotics supplementation and subsequently challenged with *Salmonella* Typhimurium or left as a control. To understand the effects of different commercial probiotics and synbiotics on *Salmonella* Typhimurium colonisation and invasion into internal organs, culture methods were used for organ load determination. The outcome of the study has broadened our understanding of the interaction of *Salmonella* Typhimurium with gut microbiota in the presence or absence of probiotic and synbiotic supplements.

Materials and methods

Rearing of birds

Fertile eggs from an Isa-Brown parent flock were obtained from a local breeder farm. Following fumigation (by formaldehyde and potassium permanganate (3:1)), the eggs were incubated in clean conditions for hatching in the School of Animal and Veterinary Sciences. From the incubator, the hatching tray papers with chicks' meconium samples were processed by culture methods for *Salmonella* isolation. Before the chicks' placement, the entire experimental facility was cleaned and then tested for *Salmonella*. The hatched chicks were reared in a house with strict biosecurity protocols as per the protocol of the ISA General Management Guide 2009-10. Next, the chicks (n = 90) were equally divided into ten treatment groups (Table 1) and reared in pens (different treatment groups in separate rooms) with water and feed provided ad libitum. The feed was fumigated as described previously and the drinking water was autoclaved. The fumigated feed was routinely tested by culture method for the presence of *Salmonella* spp. The commercially available probiotics and synbiotics used in this study were selected based on their claimed efficacy for control of *Salmonella* through gut microbiota modulation in poultry. These products are usually used in layer industry for improving birds' performance.

Four commercial probiotic and synbiotic products were purchased from the market and used in this study. For all the probiotic and synbiotic treatment groups, 1 g of each of the product was mixed in either 1 kg of fumigated feed or 1 L of autoclaved water. Every day, a freshly prepared batch of the products was offered to the treatment groups from the day of hatch to day 7 of chicks' age. At day 8 of chicks' age, birds from the probiotics and synbiotics supplemented and *Salmonella* Typhimurium treatment and positive control groups (Table 1) were challenged via the oral route with *Salmonella* Typhimurium phage type 9. The probiotics, synbiotics and negative control groups received phosphate buffered saline (PBS) only.

Inoculum preparation and birds challenge

Salmonella Typhimurium previously isolated from a layer farm (Gole et al. 2014) was used in this study. *Salmonella* Typhimurium inoculum was prepared by following the method described previously (McWhorter and Chousalkar 2018). In the challenge groups, each bird received an oral dose of 10^3 colony forming unit (CFU; 0.1 mL) of *Salmonella* Typhimurium. The inoculation dose was kept low to understand its effects on gut microbiota modulation and colonisation of internal organs. Studies suggest that approximately 10^3 CFU of *Salmonella* per chicken is enough to activate the host immune system (Chart et al. 1992; Marcq et al. 2011). A 100 μL of the original inoculum with serial dilutions was plated onto Xylose Lysine Deoxycholate (XLD; Thermo Fisher Scientific, Victoria, Australia) media to confirm the CFU received by each bird. From each treatment group at each timepoint (days 3, 5 and 7 post-infection), three birds were processed by cervical dislocation for collection of caecal contents and tissues. Previous study showed a minor variation in gut microbiota between individual birds (Videnska et al. 2014). Number of other studies also used three birds at each timepoint for gut microbiota analysis (Juricova et al. 2013; Kubasova et al. 2019).

Salmonella Typhimurium enumeration in tissue

Pieces of liver, spleen and caecal tissues were aseptically collected and weighed into 1.5 mL Eppendorf tubes containing stainless steel beads 0.5–2.0 mm and PBS. The samples were maintained on ice until further use. Tissues were homogenised using a Bullet Blender Storm homogeniser (Next Advance, NY, USA) on full speed for 5–10 min. Serial dilutions were prepared from the original tissue homogenates, plated onto XLD agar and incubated overnight at 37 °C. The *Salmonella* Typhimurium colonies were counted to determine the bacterial load ($\log_{10}$ CFU) in 1 g of tissue. A 100-μL sample from the original homogenates was also enriched in 900 μL buffered peptone water (BPW; Thermo Fisher, Victoria, Australia) and incubated overnight at 37 °C. A 100-μL sample of the incubated BPW samples was added to 10 mL of Rappaport Vassiliadis soya peptone (RVS) broth (Thermo Fisher Scientific, Victoria, Australia) and incubated overnight at 42 °C for selective growth of *Salmonella*. Suspected *Salmonella* cultures from XLD agar were sub-cultured on Brilliance *Salmonella* agar (BSA; Thermo Fisher Scientific, Victoria, Australia) plates for confirmation. A 100 mg of each of the probiotics and synbiotics was cultured in De Man, Rogosa and Sharpe (MRS) media and characteristic colonies were gram stained. For approximate CFU count in 1 g of the probiotics and synbiotics, a 100 mg of individual products was suspended into PBS, serially diluted and plated (100 μL) on MRS media.

Table 1 Treatment group distribution and probiotics/synbiotics details used in the study

Code used for supplement	Commercial product composition	Aerobically grown CFU	Anaerobically grown CFU	Mode of administration	Treatment group[a]
Probiotic A	*Lactobacillus acidophilus*, *L. delbreuckii* subspecies *bulgaricus*, *L. plantarum, L. rhamnosus, Bifidobacterium bifidum, Enterococcus faecium* and *Streptococcus salivarius* subspecies *thermophilus*	11.008	8.411	Water	Probiotic A control Probiotic A and *ST*[b] challenge
Probiotic B	*Bacillus subtilis* strains and *Bacillus amyloliquefaciens*	8.204	4.040	Feed	Probiotic B control Probiotic B and *ST* challenge
Synbiotic A	*Enterococcus* sp., *Pediococcus* sp., *Bifidobacterium* sp., *Lactobacillus* sp. and fructooligosaccharides	4.944	2.954	Water	Synbiotic A control Synbiotic A and *ST* challenge
Synbiotic B	*Lactobacillus acidophilus, L. casei, L. salivarius, L. plantarum, L. rhamnosus, L. brevis, Bifidobacterium bifidum, B. lactis, S. thermophiles*, prebiotic inulin (chicory root extract), protease, amylase, cellulase, hemicellulase, lipase, papain and bromelain	8.602	8.049	Water	Synbiotic B control Synbiotic B and *ST* challenge
Controls in the study					Positive control (normal feed and *ST* challenge) Negative control (normal feed, no *ST* challenge)

[a] In each treatment group, there was a total of 9 chicks

[b] *ST* is *Salmonella* Typhimurium. Each product was aerobically and anaerobically grown on MRS media to understand the approximate $\log_{10}$ CFU per gram of the product

Caecal contents DNA extraction

To obtain a quality DNA from caecal contents, the manufacturer's protocol (QIAamp DNA Stool Mini Kit; Qiagen, Victoria Australia) was modified slightly. Briefly, approximately 180 mg of caecal contents was weighed into a 1.5-mL tube (Eppendorf Safe-Lock; Eppendorf, Hamburg, Germany). Glass beads (acid-washed ≤ 106 μm and 425–600 μm; Sigma Aldrich, St. Louis, MO) were added to the samples and maintained on ice. Next, the samples were processed for DNA extraction as per the kit protocol except for the inclusion of the step of homogenisation in a bullet blender. DNA was eluted in 100 μL of buffer ATE (10 mM Tris-Cl pH 8.3, 0.1 mM EDTA and 0.04% NaN3) as per protocol of QIAamp DNA Stool Mini Kit. The purity (260/280 ratio 1.70–2.05; 230/260 ratio 1.80–2.30) and concentrations (20~300 ng/μL) were measured in a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Victoria, Australia). The DNA samples were stored at − 80 °C until used for downstream applications. Three samples at each sampling time-point per treatment group were submitted to the Australian Genome Research Facility (Melbourne, Australia) for diversity profiling analysis (16S: 341F–806R (V3–V4)) using the forward (CCTAYGGGRBGCASCAG) and reverse (GGACTACNNGGGTATCTAAT) primer pair.

16S rRNA analysis

PCR amplification and MiSeq sequencing

PCR amplicons were generated using the primers designed from V3–V4 region amplification of bacterial DNA using AmpliTaq Gold 360 master mix (Thermo Fisher Scientific, Victoria, Australia) for the primary PCR. The PCR conditions were initial heating at 95 °C for 7 min and 29 cycles of dissociation at 94 °C for 30 s, annealing at 50 °C for 60 s and extension at 72 °C for 60 s with a final finish of 72 °C for 7 min. A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Takara Bio, CA, USA). The resulting amplicons were measured by Qubit 4 fluorometer (Thermo Fisher Scientific, Victoria, Australia) and normalised. The equimolar pool was then

measured by qPCR (KAPA) followed by sequencing on the Illumina MiSeq Platform (San Diego, CA, USA) with 2 × 300 base pairs paired-end chemistry.

Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al. 2013). From the sequences, primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (qiime 1.8) (Caporaso et al. 2010), USEARCH (version 7.1.1090) (Edgar 2010; Edgar et al. 2011) and UPARSE (Edgar 2013) software. Using USEARCH, sequences were quality filtered, and full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using "rdp_gold" database as a reference. To obtain the number of reads in each operational taxonomic unit (OTU), sequences were mapped back to OTUs with a minimum identity of 97%. In qiime, taxonomy was assigned using GreenGenes database (version 13_8, Aug 2013) (DeSantis et al. 2006). The OTU file was uploaded into Calypso software (version 8.72) (Zakrzewski et al. 2016) and the data were further analysed for group comparisons at false discovery rate (FDR) < 0.05. During data analysis in the Calypso, the OTU table was filtered to exclude taxa with low abundance (< 0.01%) and was total sum normalised (TSS) square root transformed. The processed data in the Calypso were subsequently used for univariate (one- and two-way ANOVA), multivariate and diversity analyses. The Shannon index was used to calculate the microbial alpha diversity affected by probiotics/synbiotics treatment and sampling time-point (or birds' age) at OTU, genus and family levels. In the Calypso, the Shannon index at OTU level measures how the microbes are balanced and how species (evenness) are at a similar or dominant level to each other.

Statistical analysis

The *Salmonella* Typhimurium load ($\log_{10}$ CFU) per gram of tissue was analysed in Statview v.5.0.1.0 by taking sampling time-point and treatment group as the main effects. Repeated measure analysis was used to investigate the effects of time-point or treatment on *Salmonella* load in the organs. Level of significance was determined by PLSD at $P < 0.05$.

Availability of data and materials

The metagenome raw dataset supporting the results of this study have been deposited at the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA) under the BioProject Accession Number PRJNA548995.

Results

Before the placement of chicks, the experimental facility tested was negative for *Salmonella* spp. Similarly, the fumigated feed tested was also negative for the presence of *Salmonella*. No *Salmonella* was recovered from the chicks' meconium or from chicks sampled at regular intervals until the selected groups were challenged with *Salmonella* Typhimurium (*S*T). The faecal samples collected from the challenged groups on days 3, 5 and 7 post-infection (p.i.) were positive for *Salmonella* by culture method. All the control groups were negative for *Salmonella* throughout the experiment. No mortality or clinical signs of salmonellosis were observed after the chicks were challenged with *S*T. However, during sample collection, some of the challenged birds showed partially emptied caeca with mucous plugs. On day 3 p.i., one bird from the positive control group showed necrotic foci on the liver, while one bird from the probiotic A supplemented and *S*T challenge group showed haemorrhage in the spleen. The characteristic clinical signs observed in the caeca of the *S*T challenged groups have been summarised in Table 2.

Gut microbiota sequencing and quality of generated data

A total of 6.45 Gb sequences data for the ten different treatment groups were generated using Illumina sequencing. The

Table 2 Gross lesions observed in the caeca of different treatment groups

Treatment group[a]	Day post-infection	Lesion observed	% of bird affected
Positive control	3	Partially filled caeca and mucous plug	33
Positive control	5	Partially filled caeca	33
Positive control	7	Partially filled caeca	66
Probiotic A and *S*T[b] challenge	7	Mucous plug	33
Probiotic B and *S*T challenge	7	Partially filled caeca	33
Synbiotic A and *S*T challenge	5	Partially filled caeca	33

[a] Details of the treatment groups have been provided in Table 1

[b] *S*T is *Salmonella* Typhimurium

average reads numbers per treatment group and quality have been tabulated in Table 3. To further check the depth of microbial communities' coverage, a rarefaction analysis curve was calculated for all the treatment groups. In rarefaction analysis, the number of observed species was counted and plotted as a function of the number of sampled sequences. The slope of the curve indicates how well the sequenced data represent the underlying microbial community. The rarefaction analysis curve showed that the underlying microbial communities in all the treatment groups were well covered by the sequenced data (Supplementary file Fig. S1).

Caecal luminal microbial communities

A total of 22 known genera was identified with some sequences reads mapped to unclassified bacterial families (Fig. 1). At family level, the communities of caecal bacteria mainly comprised of *Bacillaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Paenibacillaceae*, *Peptostreptococcaceae*, *Pseudomonadaceae*, *Ruminococcaceae*, *Streptococcaceae* and *Veillonellaceae* in addition to unclassified bacteria. The three most abundant phyla were *Actinobacteria*, *Firmicutes* and *Proteobacteria*.

Caecal luminal bacterial abundance and diversity are affected by probiotic and synbiotic supplementation and *Salmonella* Typhimurium challenge

Overall, the probiotic and synbiotic supplementation and *S*T challenge significantly (FDR < 0.05) affected microbial abundance in the caecal lumen of layer chicks. Within each treatment group, sampling time-point (or flock age) significantly affected ($P < 0.05$) the microbial diversity. To gain insight into microbial abundance and diversity, individual probiotic or synbiotic supplemented group samples (with or without *S*T challenge) were analysed against the positive control and negative control groups. Data from the different probiotic or synbiotic treatment groups were not compared with one another because the contents of each product were different. Only the genera and families significantly affected (FDR < 0.05) by probiotic, synbiotic treatments or *S*T challenge have been presented here.

Effect of probiotic A supplementation

At the genus level, in the probiotic A control group, the abundance of *Trabulsiella* and *Oscillospira* was higher compared to the probiotic A supplemented and *S*T challenged, *S*T negative control and *S*T positive control groups (Fig. 2a). Unclassified *Ruminococcaceae*, *Klebsiella* and *Anaerotruncus* abundance was higher in the *S*T positive control compared with the other treatment groups. *S*T challenge decreased the abundance of *Paenibacillus* and increased *Anaerotruncus* in the *S*T positive control and probiotic A supplemented and *S*T challenged groups compared with the probiotic A control and *S*T negative control groups. Compared with the *S*T positive control group, in the presence of probiotic A supplementation, *S*T challenge affected the abundance of *Coprococcus*, *Butyricicoccus*, *Eubacterium* and *Blautia* differently as seen in the probiotic A supplemented and *S*T challenged group. A complete list of the significant genera affected by the probiotic A supplementation and/or *S*T challenge is depicted in Fig. 2a. The core caecal microbiome was affected by the probiotic A supplementation and *S*T challenge (Supplementary file Fig. S2). At OTU level, there were 32 common taxa in the probiotic A supplemented group.

In the probiotic A supplemented group, at OTU level, the Shannon index showed that the bacterial communities per group were at the same level ($P = 0.6600$) across all the treatment groups (Supplementary file Fig. S3). Within each treatment group, bird age (or sampling time-points) increased ($P = 0.0230$) the diversity of microbial communities in the

Table 3 Reads quality generated in the study

Treatment group	Raw average reads	After QC (average)	Mapped at 97%
Negative control	118,632.00	93,701.22	90,890.19
Positive control	143,071.40	111,496.10	108,151.20
Probiotic A control	144,692.70	112,840.90	109,455.70
Probiotic A and *S*T[a] challenge	126,533.30	93,272.33	90,474.16
Probiotic B control	102,005.40	80,460.56	78,046.74
Probiotic B and *S*T challenge	105,908.60	81,927.33	79,469.51
Synbiotic A control	75,867.67	59,892.33	58,095.56
Synbiotic A and *S*T challenge	122,043.40	95,423.89	92,561.17
Synbiotic B control	125,146.40	95,996.67	93,116.77
Synbiotic B and *S*T challenge	126,789.00	96,383.67	93,492.16

Details of individual probiotics and synbiotics have been mentioned in Table 1

[a] *S*T is *Salmonella* Typhimurium

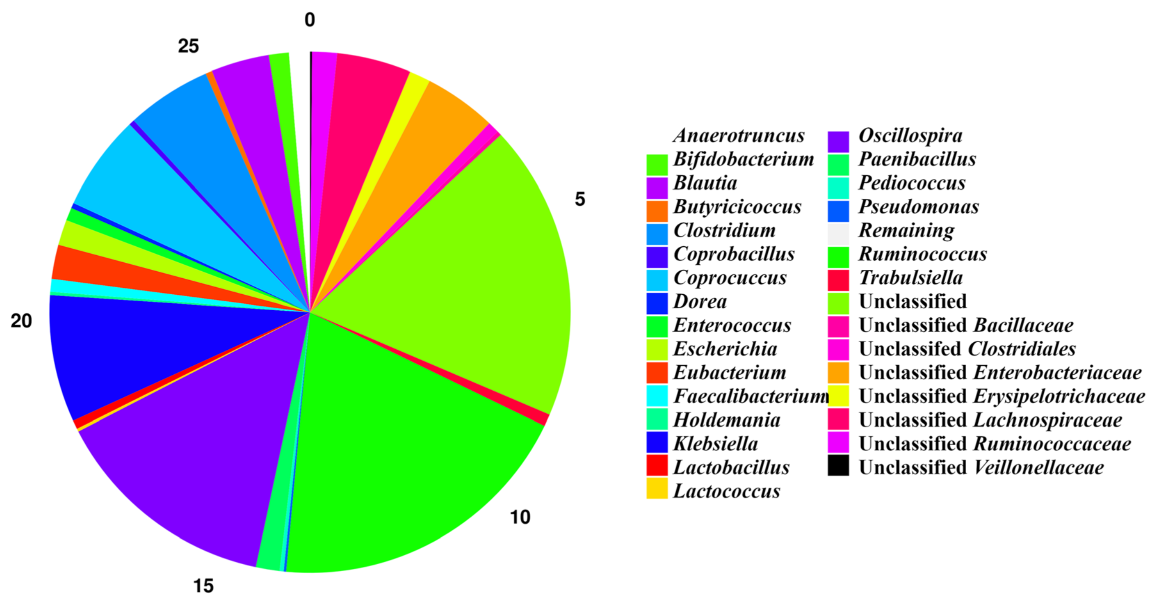

Fig. 1 Overall genera observed in the caecal lumen of layer chicks. For genera taxa visualisation, OTU data were clustered in all the treatment groups combined in Calypso software

negative control ($P = 0.0091$) and the probiotic A control ($P = 0.0046$) groups, while *ST* challenge with ($P = 0.2723$) or without ($P = 0.3128$) probiotic A supplementation had no significant effect on the microbial diversity (Fig. 2b). The Shannon index at the genus and family level produced very similar results to OTU level diversity measurements. The

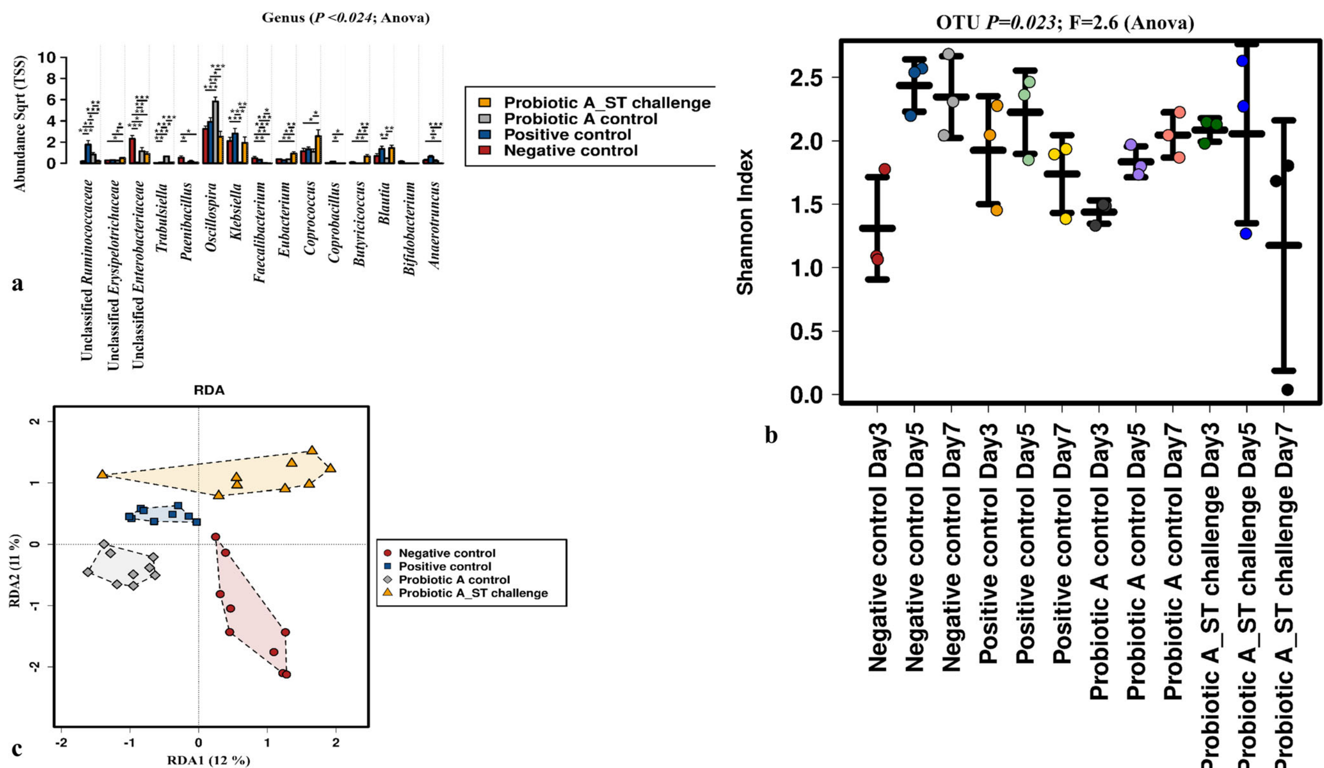

Fig. 2 Caecal luminal microbial abundance and diversity affected by probiotic A supplementation and *Salmonella* Typhimurium (*ST*) challenge. **a** Microbial abundance at genera level. **b** Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point). **c** RDA+ of each of the treatment group. $P < 0.024$ was equivalent of FDR < 0.05 in Calypso software. Probiotic A was comprised of *Lactobacillus acidophilus*, *L. delbrueckii* subspecies *bulgaricus*, *L. plantarum, L. rhamnosus, Bifidobacterium bifidum, Enterococcus faecium* and *Streptococcus salivarius* subspecies *thermophilus*

redundancy analysis (RDA+) is a multivariate method that is used to explore complex associations between community composition and multiple explanatory variables. The RDA+ showed that overall the treatment groups clustered separately showing the significant effects (P = 0.001) of probiotic A supplementation and *ST* challenge on the composition of bacterial communities (Fig. 2c).

Effect of probiotic B supplementation

Probiotic B supplementation and *ST* challenge had a significant effect (FDR < 0.05) on caecal luminal microbial abundance at the genus and family levels. At the genus level, *ST* challenge increased the abundance of unclassified bacteria and decreased *Ruminococcaceae* both in the probiotic B supplemented and *ST* challenged and *ST* positive control groups (Fig. 3a). The abundance of *Ruminococcus* and *Bifidobacterium* was higher in the probiotic B supplemented control group compared with the probiotic B supplemented and *ST* challenged, *ST* positive control and negative control groups. *Paenibacillus*, *Lactococcus*, *Eubacterium*, *Enterococcus* and *Coprobacillus* were abundant in the probiotic B supplemented and *ST* challenged group compared with the probiotic B control, *ST* positive control and negative control groups. The core caecal microbiome was affected by the probiotic B supplementation and *ST* challenge (Supplementary file Fig. S4). At OTU level, there were 30 common taxa in the probiotic B supplemented group.

Probiotic B supplementation and *ST* challenge did not significantly affect (P = 0.0860) microbial diversity among the treatment groups (Supplementary file Fig. S5). Alpha diversity measured by the Shannon index based on sampling time-point (or birds' age) showed a significant (P = 6e^{-05}) variation in microbial diversity (Fig. 3b). Probiotic B supplementation reduced (P = 0.0032) the microbial diversity on day 5 and day 7 compared with the day 3 in the probiotic B control group, although it was not altered (P = 0.1252) in the probiotic B supplemented and *ST* challenged group. The RDA+ showed that probiotic B supplementation and *ST* challenge changed (P = 0.001) the composition of bacterial communities (Fig. 3c).

Effect of synbiotic A supplementation

At the genus level, in the synbiotic A supplemented and *ST* challenge group, the abundance of *Anaerotruncus*, *Lactococcus*, *Lactobacillus*, *Eubacterium* and *Bifidobacterium* was significantly (FDR < 0.05) higher compared with the synbiotic A control, *ST* positive control and negative control

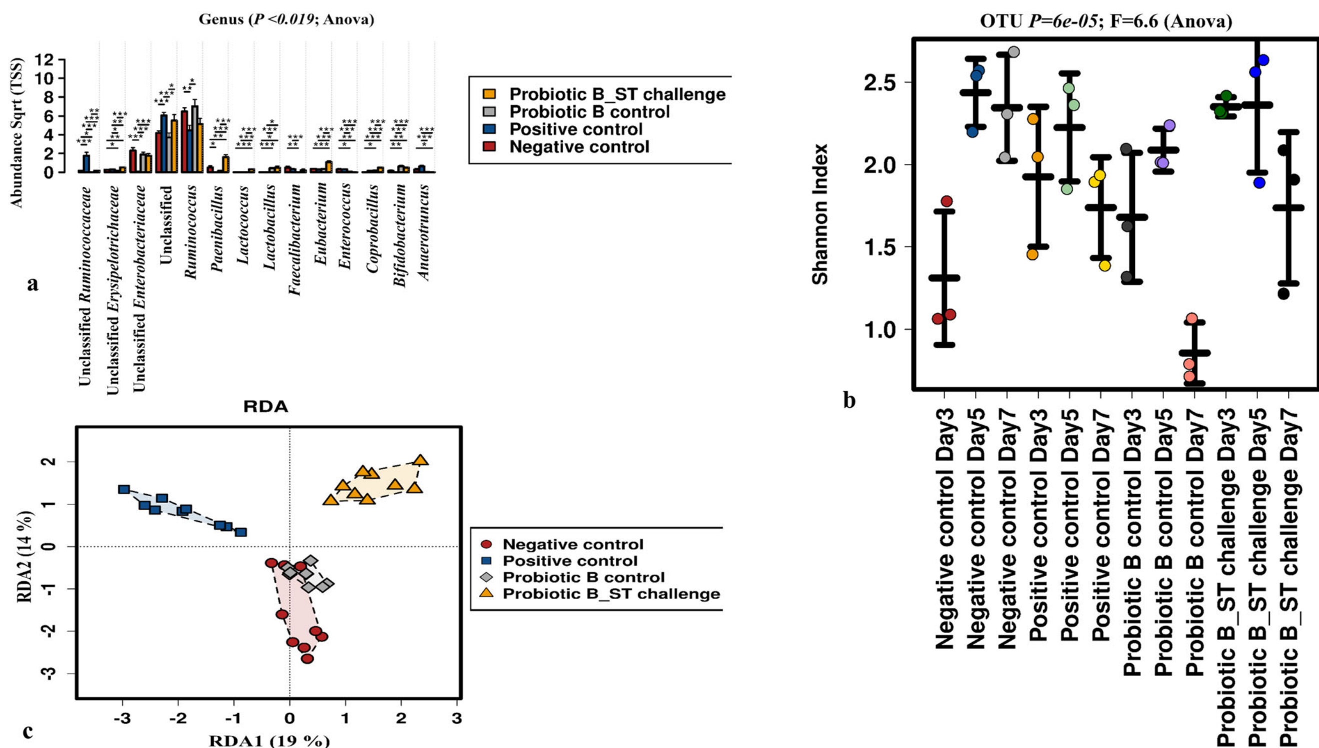

Fig. 3 Caecal luminal microbial abundance and diversity affected by probiotic B supplementation and *Salmonella* Typhimurium (*ST*) challenge. **a** Microbial abundance at genera level. **b** Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point). **c** RDA+ of each of the treatment group. P < 0.019 was equivalent of FDR < 0.05 in Calypso software. Probiotic B contained *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* in its composition

groups (Fig. 4a). Synbiotic A supplementation without *S*T challenge significantly increased the abundance of *Trabulsiella* and *Ruminococcus* as seen in the synbiotic A control group. A list of all of the significant genera has been provided in Fig. 4a. The core caecal microbiome was mainly affected by the synbiotic A supplementation interaction with *S*T challenge (Supplementary file Fig. S6). At OTU level, there were 28 common taxa in the synbiotic A supplemented group. There were no specific taxa attributed to the synbiotic A control group.

A significant effect (P = 0.0072) of synbiotic A supplementation was observed on microbial diversity in different treatment groups (Supplementary file Fig. S7). The microbial diversity was significantly lower in the synbiotic A control group compared with the synbiotic A supplemented and *S*T challenged, *S*T positive control and negative control groups. Within each treatment group (P = 0.0012), microbial diversity significantly decreased (P = 0.0191) with bird age (or sampling time-point) in the synbiotic A control and negative control groups, while in the synbiotic A supplemented and *S*T challenged (P = 0.4262) and *S*T positive control (P = 0.3128) groups, there was no difference in the microbial diversity (Fig. 4b). The RDA+ showed that synbiotic A supplementation and *S*T challenge changed (P = 0.001) the composition of bacterial communities, although some overlap between the synbiotic A control and negative control groups was observed (Fig. 4c).

Effect of synbiotic B supplementation

At the genus level, in the synbiotic B control group, the abundance of *Trabulsiella*, *Oscillospira* and *Holdemania* significantly increased compared with the synbiotic B supplemented and *S*T challenged, *S*T positive control and negative control groups (Fig. 5a). *Escherichia* and *Coprococcus* genera were higher in abundance in the synbiotic B supplemented and *S*T challenged group compared with the other three treatment groups. Overall, the *S*T challenge increased the abundance of unclassified bacteria in the *S*T positive control group. The overall genera significantly affected by the synbiotic B supplementation and *S*T challenge have been depicted in Fig. 5a. The core caecal microbiome was affected by the synbiotic B supplementation and *S*T challenge (Supplementary file Fig. S8). At OTU level, there were 36 common taxa in the synbiotic B supplemented group.

In the synbiotic B supplemented group, at the OTU level, the diversity of the microbial communities was not affected (P = 0.4200) by the synbiotic supplementation or *S*T challenge (Supplementary file Fig. S9). Overall, sampling time-point (or birds age) had a significant effect (P = 0.004) on microbial diversity (Fig. 5b) only in the negative control group (P = 0.0091). The RDA+ showed that synbiotic B supplementation and *S*T challenge changed (P = 0.001) the composition of bacterial communities (Fig. 5c).

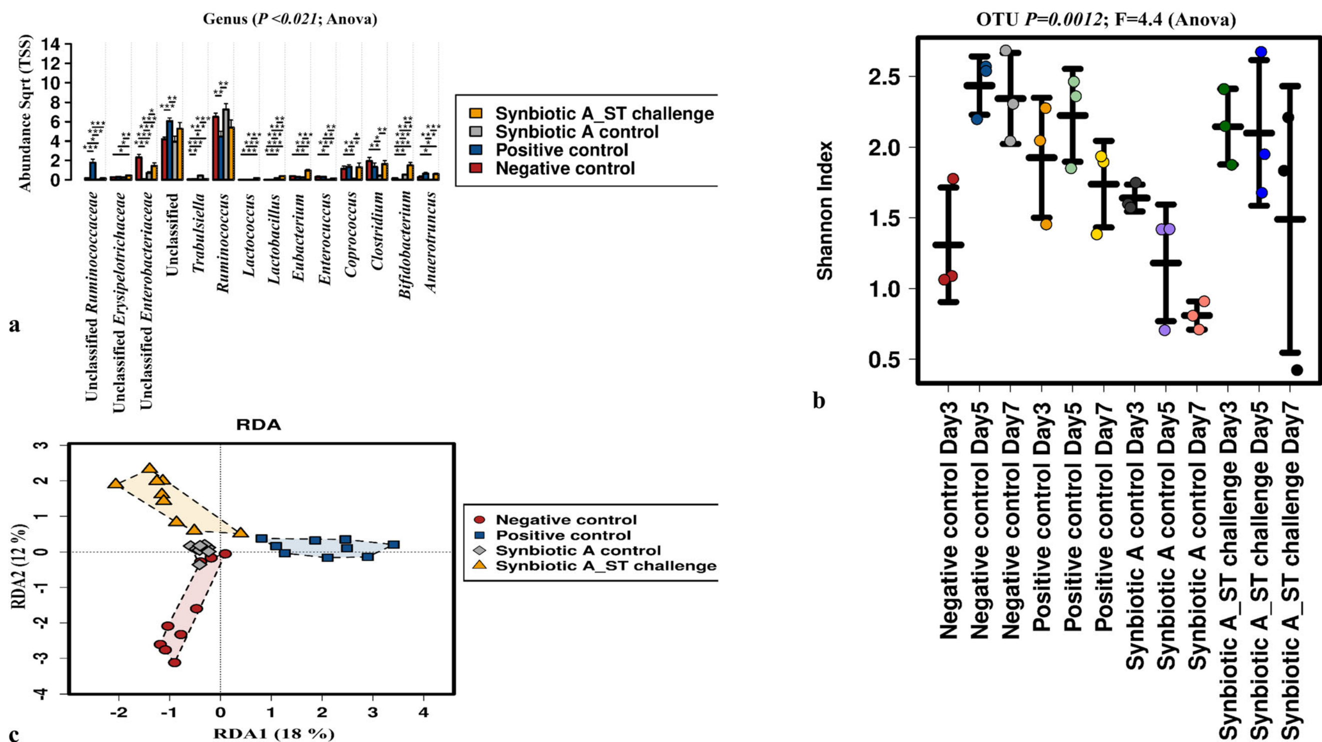

Fig. 4 Caecal luminal microbial abundance and diversity affected by the synbiotic A supplementation and *Salmonella* Typhimurium (*S*T) challenge. **a** Microbial abundance at genera level. **b** Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point). **c** RDA+ of each of the treatment group. P < 0.021 was equivalent of FDR < 0.05 in Calypso software. Synbiotic A was composed of *Enterococcus* sp., *Pediococcus* sp., *Bifidobacterium* sp., *Lactobacillus* sp. and fructooligosaccharides

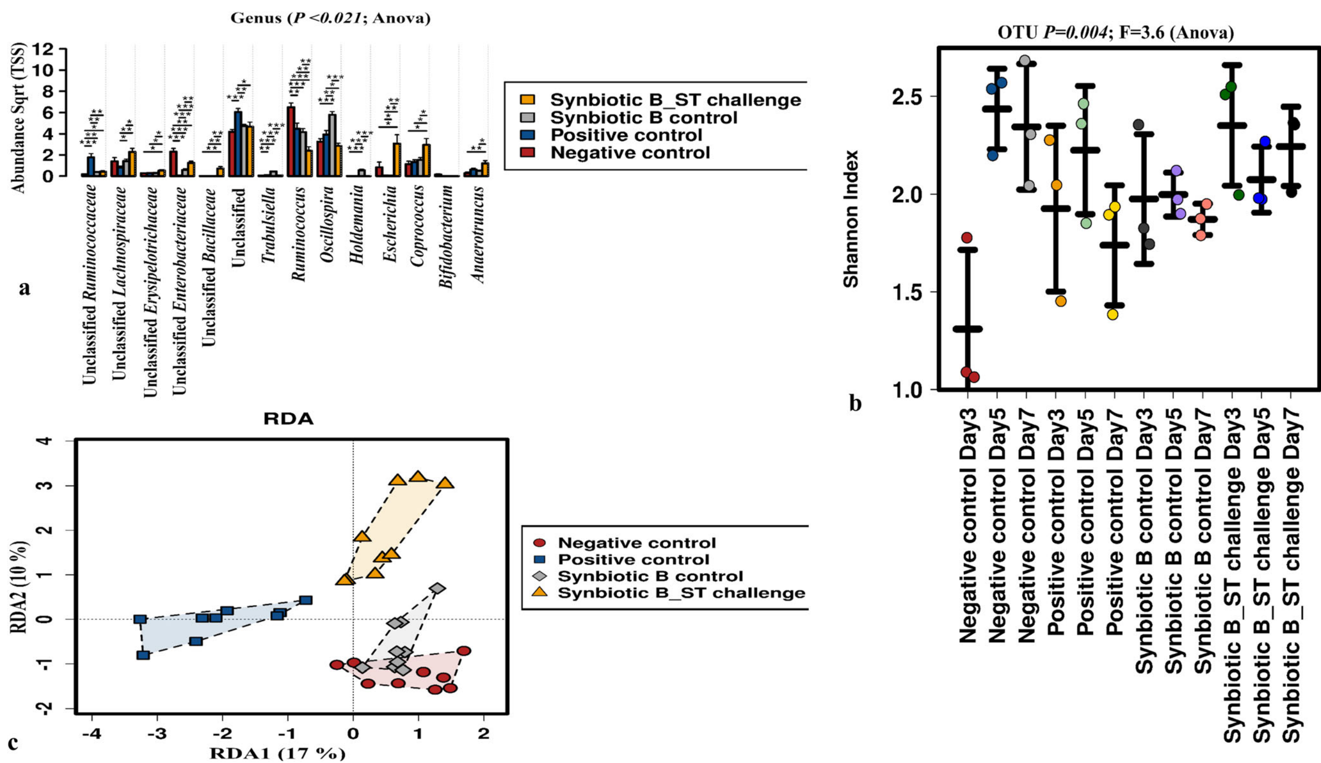

Fig. 5 Caecal luminal microbial abundance and diversity affected by synbiotic B supplementation and *Salmonella* Typhimurium (*S*T) challenge. **a** Microbial abundance at genera level. **b** Stripchart showing the Shannon index of the OTU (diversity) affected by bird age (or sampling time-point). **c** RDA+ of each of the treatment group. $P < 0.019$ was equivalent of FDR < 0.05 in Calypso software. Synbiotic B contained *Lactobacillus acidophilus, L. casei, L. salivarius, L. plantarum, L. rhamnosus, L. brevis, Bifidobacterium bifidum, B. lactis, S. thermophiles*, prebiotic inulin (chicory root extract), protease, amylase, cellulase, hemicellulase, lipase, papain and bromelain in its composition

Salmonella Typhimurium load in different organs

Salmonella spp. were not recovered from either the negative control group or the probiotics and synbiotics control groups. Probiotics A and B supplementation in young layer chicks for a week did not significantly reduce ($P > 0.05$) the *S*T load ($\log_{10}$ CFU/g of tissue) in the caeca, liver and spleen (Fig. 6a–f). In the probiotics A and B supplemented and challenged with *S*T groups, the bacterial load in all three organs increased with day p.i. except for the caeca and liver of the probiotic A challenged group (Fig. 6a–f).

Feeding synbiotics A and B to layer chicks for a week did not significantly reduce ($P > 0.05$) *S*T load in the caeca, liver and spleen. The *S*T load significantly increased with day p.i. only in the caeca (Fig. 7a–f). There was a general trend of lower *S*T load in the liver and spleen of the synbiotics A and B supplemented groups; however, these differences were not statistically significant.

Discussion

This study aimed to understand the effects of short-term feeding of four different commercial probiotic and synbiotic supplements on gut health in the presence or absence of *Salmonella* Typhimurium challenge in Isa-Brown layer chicks. During the last century, the incorporation of growth-promoting antibiotics into the feed of production animals has resulted in improvements to health conditions and productivity. Globally there are concerns about the use of in-feed antibiotics for growth promotion due to the development of antimicrobial resistance and the spread of resistance genes. Moreover, the use of antimicrobials can negatively alter gut microbiota. Probiotics and synbiotics are favoured in recent days due to their ability to induce a structural change in gut microbiota.

The rationale behind the discontinuation of the probiotics and synbiotics before *Salmonella* Typhimurium challenge was to study the effects of the *Salmonella* infection on developed gut microbiota and *Salmonella* invasion into internal organs. In this study, irrespective of the probiotic and synbiotic supplementation and *Salmonella* Typhimurium challenge, a total of 22 distinct genera were identified in layer chicks; however, we have only discussed the genera significantly affected by the probiotics, synbiotics or *Salmonella* Typhimurium challenge relative to positive and negative control groups.

In the current study, most of the genera that colonised the chicks' caeca are involved in diverse physiological functions. For example, *Bifidobacterium* (Milani et al. 2015), *Clostridium* (Bayer et al. 2008), *Enterococcus* (Robert and Bernalier-Donadille 2003), *Eubacterium* (Montgomery

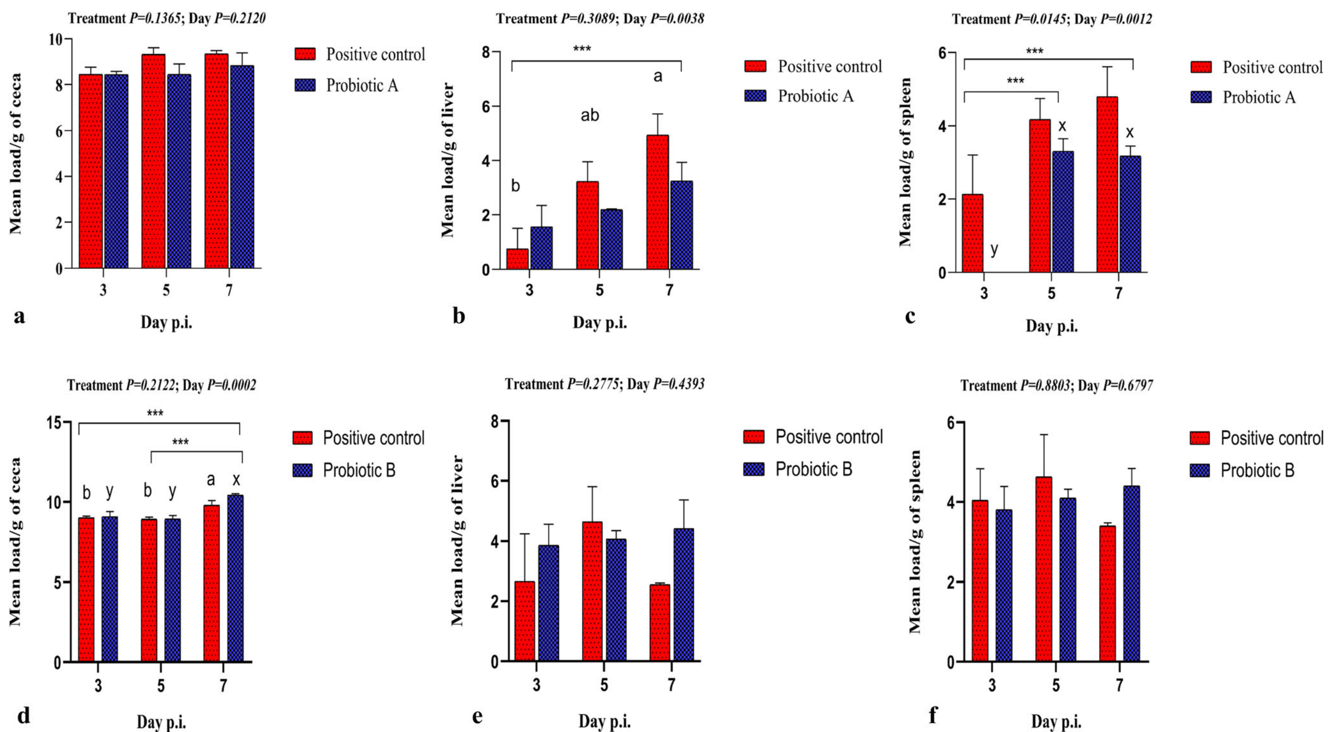

Fig. 6 Effect of probiotics A and B on mean load (log_{10} CFU/g of tissue) of *Salmonella* Typhimurium (*ST*) in caeca, liver and spleen collected on days 3, 5 and 7 p.i. **a** *ST* load in caecal tissue of probiotic A supplemented chicks. **b** *ST* load in liver tissue of probiotic A supplemented chicks. **c** *ST* load in spleen tissue of probiotic A supplemented chicks. **d** *ST* load in caecal tissue of probiotic B supplemented chicks. **e** *ST* load in liver tissue of probiotic B supplemented chicks. **f** *ST* load in spleen tissue of probiotic B supplemented chicks. Superscripts (^{a,b}) represent significant difference affected by day p.i. in positive control group. Superscripts (^{x,y}) represent significant difference affected by day p.i. in probiotic supplemented group. In each graph, line across the bars represents significant differences (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$) between the respective groups. Details of probiotics A and B are in Table 1

1988) and *Ruminococcus* (Moon et al. 2011) in the gut are involved in fibre digestion. *Eubacterium* is one of the dominant genera of the caecum in layers (Callaway et al. 2009). Some species of *Eubacterium* (for example *Eubacterium hallii*) in the gut are involved in metabolising glycerol to 3-hydroxypropionaldehyde that exists in a multi-compound system called reuterin (Fekry et al. 2016). Reuterin possesses antimicrobial activity against a range of pathogens (Vollenweider et al. 2010). *Eubacterium hallii* in the human gut has been linked with the formation of propionate (Engels et al. 2016). Therefore, the most abundant *Eubacterium* in chicks' caeca could be linked with numerous functions ranging from short-chain fatty acids (SCFAs) production to metabolic balance. In this study, a reduction of *Eubacterium* abundance in the *Salmonella* Typhimurium positive control group compared with the probiotics (A and B) and synbiotic A supplemented and *Salmonella* Typhimurium challenged groups showed that these products were effective in maintaining the *Eubacterium* population in the gut even in the presence of *Salmonella* Typhimurium. This study also demonstrated some interaction between *Eubacterium* and *Salmonella* Typhimurium because, in the absence of challenge, the abundance of *Eubacterium* was not significantly different among the treatment groups.

Oscillospira is another genus of gut microbiota abundantly present in layer chickens' caeca (Volf et al. 2016*)*. *Oscillospira* species help in starch digestion in many different hosts (Mackie et al. 2003) and therefore are butyrate producers (Gophna et al. 2017). Butyrate is one of the three main types of SCFAs and is involved in ATP provision to enterocytes (Treem et al. 1994) and possesses anti-inflammatory (Vinolo et al. 2011) and antimicrobial properties (Cox et al. 1994). In this study, an increased abundance of *Oscillospira* in the probiotic A and synbiotic B supplemented groups without *Salmonella* Typhimurium challenge indicates that these products favoured starch digestion and SCFAs producing bacteria were favoured. It was also observed that, in the presence of the probiotic A and synbiotic B supplementation, *Salmonella* Typhimurium reduced the *Oscillospira* abundance compared with the *Salmonella* Typhimurium positive control group. It is possible that there is an interaction between *Oscillospira* and *Salmonella*, where *Salmonella* Typhimurium depletes its population. *Ruminococcus* is among other genera involved in SCFAs production in chickens (Huang et al. 2018). In this study, different probiotics and synbiotics affected *Ruminococcus* abundance differently. For example, synbiotic B supplementation reduced *Ruminococcus* abundance in the synbiotic supplemented groups with or without

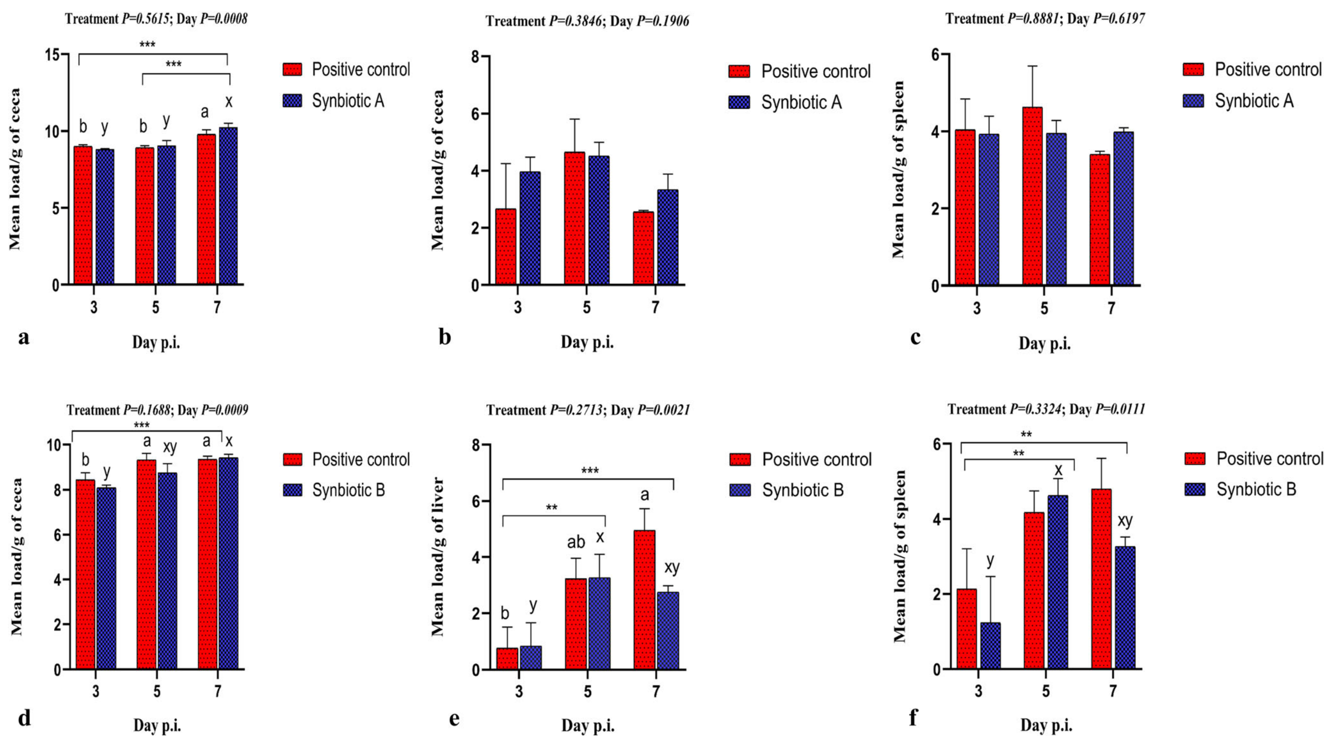

Fig. 7 Effect of synbiotics A and B on mean load ($\log_{10}$ CFU/g of tissue) of *Salmonella* Typhimurium (*ST*) in caeca, liver and spleen tissues collected on days 3, 5 and 7 p.i. **a** *ST* load in caecal tissue of synbiotic A supplemented chicks. **b** *ST* load in liver tissue of synbiotic A supplemented chicks. **c** *ST* load in spleen tissue of synbiotic A supplemented chicks. **d** *ST* load in caecal tissue of synbiotic B supplemented chicks. **e** *ST* load in liver tissue of synbiotic B supplemented chicks. **f** *ST* load in spleen tissue of synbiotic B supplemented chicks. Superscripts (^{a,b}) represent significant difference affected by day p.i. in positive control group. Superscripts (^{x,y}) represent significant difference affected by day p.i. in probiotic supplemented group. In each graph, line across the bars represents significant differences (***P < 0.0005; **P <0.005) between the respective groups. Details of synbiotics A and B are in Table 1

Salmonella Typhimurium challenge. Probiotic B and synbiotic A increased the *Ruminococcus* abundance both in the probiotic and synbiotic supplemented and *Salmonella* Typhimurium challenged groups. This shows that one particular probiotic or synbiotic may not favour the abundance of all beneficial bacterial genera in the gut. In this study, the predominant bacterial genera of *Eubacterium*, *Oscillospira* and *Ruminococcus* positively modulated by the probiotics and synbiotics indicate their role in the overall gut health in young chicks. No significant effect of *Salmonella* Typhimurium and *Salmonella* Enteritidis was observed on the composition of gut microbial communities of laying chickens (Azcarate-Peril et al. 2018; Nordentoft et al. 2011).

Probiotic A and synbiotics A and B supplementation increased the abundance of *Trabulsiella*, while *Salmonella* Typhimurium challenge decreased it. *Trabulsiella* is a member of the *Enterobacteriaceae* with no apparent role in chicken gut microbiota. *Bifidobacterium* is one of the dominant members of gut microbiota that plays a role in complex starch digestion (Milani et al. 2015), preventing the production of proinflammatory cytokines (Fanning et al. 2012) and stress reduction (Savignac et al. 2014). In this study, its abundance was affected mainly by the probiotic B and synbiotic A with or without *Salmonella* Typhimurium challenge. These probiotic and synbiotic were effective in maintaining *Bifidobacterium* abundance even in the presence of *Salmonella* Typhimurium. This shows that *Bifidobacterium* is one of the gut bacteria with a possible protective role against *Salmonella* Typhimurium infection. However, at least in the conditions applied in the current study, the protective environment produced by the *Bifidobacterium* was not sufficient to reduce the load of *Salmonella* Typhimurium significantly in caeca. We suggest further investigation to understand the interaction of *Bifidobacterium* with *Salmonella* Typhimurium as a probiotic candidate for the chicken gut. A reduced abundance of *Klebsiella* and *Escherichia* in the probiotic A and synbiotic B supplemented and no *Salmonella* Typhimurium challenge groups, respectively, showed the positive modulation of gut microbiota by these products. Important diseases of poultry attributed to *Escherichia* include cellulitis, septicaemia, colibacillosis, omphalitis and respiratory tract infection.

Different probiotics and synbiotics supplementation during *Salmonella* Typhimurium infection showed positive effects on the abundance of certain genera. For example, probiotic A supplementation increased the abundance of *Butyricicoccus*, *Eubacterium*, *Coprococcus* and *Blautia* in the *Salmonella* Typhimurium challenged compared to the

positive control group. Similarly, synbiotic B affected the abundance of *Escherichia*, *Coprococcus* and *Anaerotruncus*, while synbiotic A affected *Lactococcus*, *Lactobacillus*, *Eubacterium*, *Coprococcus*, *Bifidobacterium* and *Anaerotruncus*. Probiotic B mainly influenced *Paenibacillus*, *Eubacterium* and *Coprobacillus*. This further strengthens the notion that, during the *Salmonella* Typhimurium infection, short-term feeding of probiotics or synbiotics has the potential to influence resident gut bacterial genera positively. However, this short-term feeding may not be sufficient to inhibit *Salmonella* Typhimurium from colonising caeca or invading internal organs. Overall, compared to the negative control group, the microbial diversity (measured at OTU level) was decreased with bird age (or sampling time-point) by synbiotic A supplementation. This shows that the synbiotic A reduced the genera of certain bacteria in the gut of layer chicks. The Shannon index of redundancy analysis (RDA+) showed that probiotic and synbiotic supplementation and *Salmonella* Typhimurium challenge shifted the gut microbiota diversity and therefore it clustered separately among different treatment groups, showing the importance of probiotic or synbiotic supplementation in *Salmonella* Typhimurium infected birds. These results were further supported by the presence of various biomarkers in different treatment groups. Probiotic and synbiotic supplementation increased the abundance of many genera of the gut microbiota in the presence or absence of *Salmonella* Typhimurium; however, these effects varied depending on the products.

The bacteriology results (counted as CFU/g of tissue) showed that feeding the probiotics and synbiotics for a week was not effective in significantly reducing *S*T load in the liver, spleen and caecal tissues of layer chicks. It seems that the early colonisation of caeca with probiotic bacterial strains may not competitively exclude *Salmonella* Typhimurium. Limited studies performed on probiotic or prebiotic supplementation and *Salmonella* Typhimurium challenge in layer chickens presented different results reflecting the importance of the nature and duration of the probiotic being supplemented, genetic strain, age of the bird and the bird rearing environment. For example, the inclusion of fructooligosaccharides (FOS) in an alfalfa moulting diet significantly decreased caecal *Salmonella* Enteritidis counts in laying hens (Donalson et al. 2008). In White Leghorn Hy-Line cockerels challenged with *Salmonella* Enteritidis, FOS supplementation (alone or in combination with probiotic) significantly decreased *Salmonella* load in caeca at days 1, 7 and 14 p.i. (Fukata et al. 1999). In 46-week old White Leghorn laying hens challenged with *Salmonella* Enteritidis, supplementation of *Lactobacillus plantarum* for 7 days post-infection did not significantly reduce *Salmonella* load in the caeca (Adhikari et al. 2018). Interestingly, synbiotic supplemented laying hens challenged with *Salmonella* Enteritidis showed no *Salmonella* in the caecal contents on day 10 p.i. (Luoma et al. 2017). *Lactobacillus johnsonii* strain R-17504 and *Lactobacillus reuteri* strain R-17485 supplementation into day-old layer chicks significantly reduced *Salmonella* Enteritidis load in caeca on day 6 p.i. (Van Coillie et al. 2007). In this study, no significant difference between the treatment groups for *Salmonella* Typhimurium load in caecal tissue indicate that the probiotics and synbiotics were not effective in reducing *Salmonella* count at days 3, 5 and 7 p.i. in Isa-Brown layer chicks. Further studies are required to investigate the long-term feeding of probiotic supplements on *Salmonella* Typhimurium colonisation, shedding and or invasion into vital organs of hens. Overall, a weeklong probiotic and synbiotic supplementation to layer chicks was effective in modulating positively the abundance of certain resident gut microbiota. *Salmonella* Typhimurium challenge decreased the abundance of many useful bacterial genera in chicks that were not supplemented with probiotics or synbiotic. On the other hand, there was an increase in the abundance of many useful bacterial genera in infected chicks that were supplemented with probiotics and synbiotics. These abundant genera play a pivotal role in maintaining overall gut health. Nevertheless, probiotic and synbiotic supplementation to chicks for 1 week did not competitively exclude *Salmonella* Typhimurium from caeca or prevent internal organ invasion. Further studies are required to understand the long-term feeding of probiotics on laying chicken gut microbiota and its effects on *Salmonella* Typhimurium shedding.

Acknowledgements Help in sample collection from Nitish Narendra Joat (The University of Adelaide) is acknowledged. Critical editorial comments on the manuscript by Juliet Roberts (University of New England, Australia) are highly acknowledged.

Authors' contributions K.K.C. developed the hypothesis and designed the experimental work. S.K. and K.K.C. performed the experimental work, analysed the data and wrote the manuscript. Both the authors reviewed and approved the manuscript for publication.

Funding information This study was financially supported by the Australian Eggs Limited under grant number 1FS802UA to Kapil K. Chousalkar. The funding body was not involved in the collection, analysis and interpretation of the data or writing of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

Ethics statement The Animal Ethics Committee at the University of Adelaide approved the work (approval number S-2017-080) in accordance with the guidelines specified in "Australian code for the care and use of animals for scientific purposes, 8th edition (2013)." Standard Operative Procedures were followed for caring and processing of the experimental chicks.

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