Research Note: The effect of a probiotic *E. faecium* 669 mitigating *Salmonella* Enteritidis colonization of broiler chickens by improved gut integrity

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ABSTRACT In this study, we investigate the effect of the probiotic *E. faecium* 669 strain on the gut integrity of broilers and the effect on intestinal colonization with *Salmonella* Enteritidis. In the in vivo experiment, 120-day-old broilers (Ross 308) were divided into 4 equally sized groups. Group A received the probiotic as a single dose by spray at d 18 of incubation and group B received the probiotic in the drinking water daily throughout the experiment. Group C was untreated control. Group D received the antibiotic Apramycin sulfate in the drinking water. Broilers in all four groups were challenged with *S.* Enteritidis by oral gavage at d 8 of life. From d 9 to 12, a cloacal swab was collected from all broilers for culturing on *Salmonella* selective media to determine the shedding. At d 12, birds were euthanized and *S.* Enteritidis in ceca were enumerated and intestinal samples for histology and host gene expression were collected. The group receiving the probiotic in the drinking water shed significantly less *S.* Enteritidis compared to the untreated control group at all times. The group receiving a single probiotic application before hatch showed a reduced shedding of Salmonella at d 9 and 10. *S.* Enteritidis was not detected in the ceca of the antimicrobial treated broilers. Histology of jejuni samples and host gene expression showed that intestinal integrity was enhanced by adding probiotic to the drinking water.

Overall, the study shows that pre-hatch and daily application of the probiotic strain *E. faecium* 669 reduces the colonization of broilers with *S.* Enteritidis and daily application enhances gut integrity. Application of the probiotic *E. faecium* strain can be recommended as a method to reduce the colonization of broilers with *S.* Enteritidis and enhance their gut integrity.

Key words: *Salmonella* enteritidis, *Enterococcus faecium*, probiotic, broiler, gut integrity

INTRODUCTION

Salmonella infection is one of the most important foodborne infections in humans. CDC USA estimates that 1.35 million people become sick, 26,500 hospitalized and 420 die in USA each year due to *Salmonella* infection (https://www.cdc.gov/salmonella/). *Salmonella* Enteritidis and Typhimurium are the 2 most prevalent serovars causing human infections worldwide (Rabsch et al., 2001). In industrialized countries, contaminated fresh meat and eggs are the most important sources of infections. Transmission occurs from asymptomatic carrier animals, in particular swine, broilers, and layers (Rabsch et al., 2001).

Different strategies have been used for controlling *Salmonella* in layers and broilers; vaccination, antibacterial feed additives and hygiene measures are the main efforts in many countries. Some countries have implemented successful control programs. In Sweden, *Salmonella* in broilers and layers has essentially been eradicated due to a control program (Wahlström et al., 2011). A strict control program may not be realistic in all countries and antibacterial feed additives, hygiene measures during production and decontaminants during processing of carcasses may be the methods used to reduce cases of human salmonellosis. However, due to the growing concerns of development of antimicrobial resistance the demand for alternative strategies increases.

Intestinal integrity is an important parameter affecting production parameters since intestinal inflammation causes suboptimal growth. Various defense mechanisms of the intestine protect the bird against pathogens. The defense consists of the mucus layer produced by the goblet cells, the Paneth cells producing antimicrobial molecules, M cells expressing toll-like receptors, dendritic cells presenting antigens, and tight junctions connecting

the epithelial cells tightly together (Garrett et al., 2010; Capaldo et al., 2017).

The aim of the current study was to evaluate the effect of the probiotic strain *Enterococcus faecium* 669 on intestinal colonization of broiler chickens with *Salmonella enterica* subsp. *enterica* serovar Enteritidis and determine the effect of the probiotic bacteria on selected gut integrity parameters.

MATERIALS AND METHODS

Ethical Statements

The research was approved by the Danish Animal Experiments Inspectorate (license: 2019-15-0201-01611).

Bacterial Strains and Culturing

The product GalliPro Hatch consisting of *Enterococcus faecium* 669 was used as the probiotic and *Salmonella enterica* subsp. *enterica* serovar Enteritidis SE 147 (Methner et al., 1995) was used as the challenge strain. The probiotic *E. faecium* and challenge *S.* Enteritidis strains were cultured on blood agar plates prepared with 5% calf blood in blood agar base (Oxoid, Basingstoke, UK). Blood agar plates used for isolating and counting the *E. faecium* strain was supplemented with Kanamycin sulfate (1,000 μg/mL; Merck, Darmstadt, Germany). For enumeration of *S.* Enteritidis in cecal samples from chickens, samples were cultured on XLD agar (Oxoid) supplemented with streptomycin sulfate 100 μg/mL (Merck) in the following named XLD-strep.

Hatching and Housing

Fertilized eggs of broiler type Ross 308 were incubated in 2 HEKA hatchers (America-thisted, Thisted Denmark). The incubation temperature was set at 37.7°C, humidity was approximately 65% and automatic turning was applied until d 17, after which the eggs were placed into hatching trays. Unfertilized eggs were removed from the hatchers after candling between d 10 and 14.

The newly hatched chickens were housed in 4 separate pens in one room in an animal facility. The temperature was between 23 and 25°C and a heating lamp was provided in each pen. Chickens had access to feed (commercial starter feed) and water ad libitum, dust bath, and perches.

Experimental Design

The newly hatched chickens were divided into 4 experimental groups (A-D) described in Table 1. Group A received the probiotic as a spray pre-hatch (incubated in a separate hatcher) and group B, C, and D were mock-sprayed with sterile saline at the same time point. After hatch group B received the probiotic strain in the drinking water, while group C was untreated control. Group D was treated with Apramycin sulfate (Apravet WS, Huvepharma, Antwerpen, Belgium) via drinking water at a dose of 144 mg apramycin/kg bodyweight. At d 8, all four groups were challenged with an oral dose of *S.* Enteritidis (200 μL of an overnight culture diluted to 3×10^6 colony forming units (cfu)/mL.

Application of Probiotic

For group A the *E. faecium* probiotic was applied pre-hatch at d 18 of incubation as a spray on the eggs. The lyophilized powder was dissolved in sterile isotonic saline to a final concentration of 3×10^9 cfu/mL. Each egg received approximately 50 μL of the solution by a spray from a small handheld sprayer. In group B, the probiotic was applied daily in the drinking water at a concentration of 4×10^7 cfu/mL.

Shedding of S. Enteritidis

To evaluate the shedding of *S.* Enteritidis a cloacal swab was collected daily from each chicken from d 9 to 12. The cloacal swabs were streaked directly on XLD-strep agar and a second swab was placed in 10 mL buffered peptone water (**BPW**) (Merck) and incubated overnight at 37°C for pre-incubation. The growth of *S.* Enteritidis from the direct swab was evaluated semi-quantitatively. Plates were categorized as follows: 1−20 colonies = 2, 21−100 colonies = 3, > 100 colonies = 4. When plates showed no visual growth, 100 μL of the BPW was distributed in three drops on an MRSV agar plate (Merck) and incubated overnight at 42°C. From MRSV plates with a swarming zone, the bacteria from the edge of the zone were subcultured on XLD-strep to confirm the presence of *S.* Enteritidis. When the presence of *S.* Enteritidis was confirmed after pre-incubation, the growth was categorized as 1.

Bacterial Enumeration of S. Enteritidis in Ceca at D 12

At d 12, all chickens were euthanized and one ceca was collected by sterile equipment and placed in a stomacher bag. Ceca was diluted 1:1 in sterile isotonic saline, homogenized and 10-fold diluted before spotting 3×10 μL per dilution on XLD-strep. Plates were incubated at 37°C overnight before colonies were counted.

Bacterial Translocation From Intestine to Liver

The liver was aseptically removed by sterile scissor and tweezer and stored at -20°C until analysis. Liver samples were diluted 1:1 with isotonic saline and homogenized before spreading 0.5 mL on XLD-strep, incubating overnight and subsequently counting colonies.

Table 1. Experimental design and results.

Treatment	A Probiotic spray d 18 of incubation	B Probiotic in drinking water d 1−11	C Untreated control	D Apramycin in drinking water d 8−12	P-values
Challenge d 8	6×10^5 cfu	6×10^5 cfu	6×10^5 cfu	6×10^5 cfu	
Salmonella Enteritidis count[1]					
liver	1.36[ab] ± 0.14[2]	0.84 [a] ± 0.16	1.37 [ab] ± 0.14	0.26[c] ± 0.11	$P < 0.0001$
ceca	6.48[a] ± 0.27	5.10[b] ± 0.40	6.87[a] ± 0.089	0.00[c] ± 0.00	$P < 0.0001$
Relative gene expression[1]					
Mucin-2	ND[3]	1.71 ± 0.14	1.17 ± 0.10	ND	$P = 0.0039$
Zonula occludens-1	ND	4.05 ± 1.06	1.60 ± 0.36	ND	$P = 0.0330$
Claudin-1	ND	1.04 ± 0.32	1.22 ± 0.19	ND	$P = 0.6058$
Claudin-2	ND	0.48 ± 0.086	1.36 ± 0.22	ND	$P = 0.0016$
Claudin-5	ND	2.09 ± 0.31	1.08 ± 0.09	ND	$P = 0.0036$
Occludin	ND	0.90 ± 0.09	1.02 ± 0.05	ND	$P = 0.2650$
Villus morphometrics (μm)[1]					
Villus length	ND	444.70 ± 5.78	415.3 ± 4.88	410.0 ± 6.37	$P < 0.0001$
Crypt depth	ND	61.74 ± 1.10	82.92 ± 1.40	75.74 ± 1.24	$P < 0.0001$
Villus/crypt ratio	ND	7.53 ± 0.11	5.37 ± 0.10	5.62 ± 0.09	$P < 0.0001$

[1]All values presented as mean ± SEM of the Log(cfu/g+1).
[2]Columns with different superscript ([abc]) are significantly different from each other.
[3]Not determined.

Analysis of Host Gene Expression by Real-Time Quantitative Polymerase Chain Reaction

Intestinal tissue (approximately 2−4 cm and 2 cm posterior to the diverticulum of Meckel at mid jejunum) was aseptically excised and submerged in 4 mL RNAlater (Thermo Fisher Scientific, Vilnius, Lithuania) and frozen at −20°C until analysis. Total RNA extraction from 20 mg tissue samples from group B and C, reverse transcription and RT-qPCR was done according to the protocol described in (Liu et al., 2022). All primers can be found in (Chang et al., 2020) and host genes are listed in Table 1.

Evaluation of Jejunal Morphometrics

Intestinal tissue (approximately 2−4 cm) was collected at 5 cm posterior to the diverticulum of Meckel at mid jejunum. The tissue was fixed in 4% neutral buffered formaldehyde. Intestinal samples from 10 randomly selected chickens from group B, C, and D were cut into 2 to 6 cross-sections and embedded in paraffin. Hematoxylin and eosin staining was performed according to a standard protocol.

Morphometric analysis was done on 30 intact, well-oriented villus−crypt units from 2 to 3 jejunal cross-sections per chicken, providing 300 measurements/group. Villus height (μm) was measured from the tip of the villus to the villus crypt junction, and crypt depth was defined as the depth of the invagination between adjacent villi. Crypt depth was measured from the root of villus to the lamina propria at crypt bottom (Viveros et al., 2011; Emadinia et al., 2020). The evaluation of jejunal morphometrics was blinded and all measurements were performed using the open-source software ImageJ https://imagej.nih.gov/ij/download.html.

The villus/crypt ratio was calculated. The mean villus height and crypt depth were expressed as a mean villus height per treatment group.

Additionally, the histomorphology of the jejunal cross-sections were qualitatively described.

Statistical Analysis

Hatching rates were compared using Fisher's exact test. Intergroup comparisons of numerical values (e.g., *Salmonella* counts, relative gene expression level values and histological morphometrics) were analyzed by ordinary one-way ANOVA using Tukey's multiple comparison test for post hoc pairwise comparison. Bacterial counts were normalized via log transformation ($Y = \log(y+1)$), where y is the original cfu, as some samples had no bacteria present (zero count) and log transformation of zero is impossible. Data from the *S.* Enteritidis counts, gene expression analysis, and histological morphometrics were expressed as means ±SEM. The *S.* Enteritidis shedding was compared between and within groups by a Kruskal-Wallis test using Dunn's multiple comparison test for post hoc pairwise comparison. The shedding profiles were visualized as heatmaps. All statistical analyses were done using Prism 9 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). P-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Hatchability

The hatchability was 81% and 91% in group A and B/C/D, respectively. Fisher's exact test showed no difference in hatchability ($P = 0.113$).

Shedding of S. Enteritidis

The daily shedding of *S.* Enteritidis was estimated from d 9 to 12 and the shedding dynamics is illustrated in Figure 1. The heatmaps demonstrate that daily
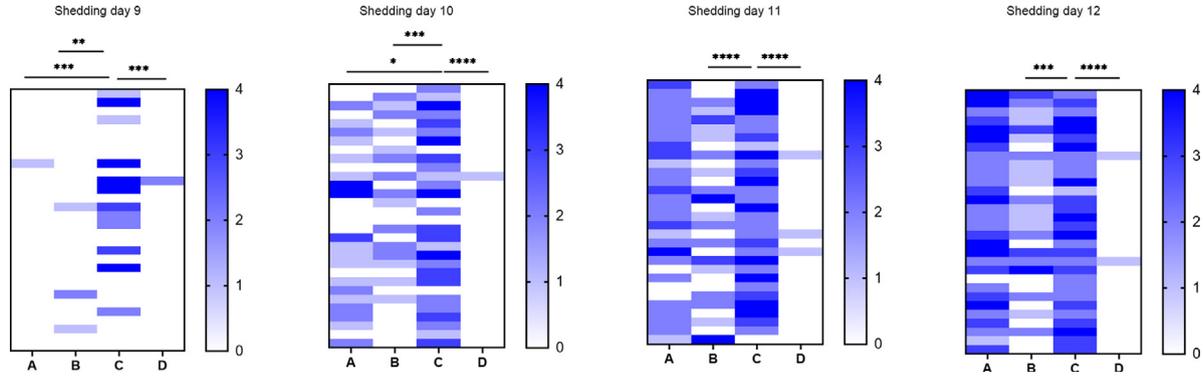
Figure 1. Shedding of *S.* enteritidis d 9 to 12. The x-axis show group A (Spray application pre hatch), B (Drinking water application of probiotic), C (untreated control) and D (Apramycin treatment). On the y-axis every chicken (n = 30) is represented by a bar. The color scale to the right shows the semi-quantitative measuring of *S.* enteritidis in the cloacal swabs. 0 = no growth, 1 = only growth from enrichment, 2 = 1−20 colonies after direct streak, 3 = 21−100 colonies after direct streak and 4 > 100 colonies. Only significant differences are shown as bars and asterisk above the heatmaps (*=<0.05, **=<0.01, ***=<0.001=. In case a comparison is non-significant the bar is not shown. Groups are compared to the untreated control group (group C).

application of probiotic in the drinking water (group B) decreased the shedding of *S.* Enteritidis at all days compared to the untreated control group (group C). Spraying probiotic pre-hatch spray (group A) reduced the shedding of *S.* Enteritidis compared to the control group (Group C) until d 10 of life, after which no effect on shedding was observed. Apramycin treatment reduced *S.* Enteritidis in ceca and after d 9 samples were only positive after pre-incubation in this group.

Bacterial Counts of S. Enteritidis

The *S.* Enteritidis cecal count (cfu) at d 12 (4 d after inoculation with *S.* Enteritidis) is shown in Table 1 as the mean log(cfu+1) ± SEM. As expected, all samples in the Apramycin treated group have bacterial counts below the detection limit. The mean *S.* Enteritidis count was lower in the group receiving the probiotic in the drinking water (group B) ($P < 0.0001$) compared to the control group (group C). No difference between the control group (group C) and the group sprayed with probiotic pre-hatch (group A) could be detected. The *S.* Enteritidis counts in ceca showed a similar distribution as the cloacal shedding.

Bacterial Translocation From Intestine to Liver

The *S.* Enteritidis count in the liver was measured to describe the translocation of bacteria from the intestine to the liver. The bacterial translocation is used as a proxy for the health status of the intestine by describing disruption of the epithelial lining of the gastrointestinal tract (Capaldo et al., 2017). The bacterial counts are also shown in Table 1 (mean log(cfu±1) ± SEM). The group receiving probiotic in the drinking water (group B) showed a lower mean count compared untreated control group (group C). Group B was not significant different from group C and again, the Apramycin treated group showed the lowest level of *S.* Enteritidis count. However

as many producers aim at reducing antimicrobial usage this treatment should be avoided.

Host Gene Expression

Relative gene expression of tight junction related genes in the control group (group C) and the probiotic treated group (group B) are also shown in Table 1. The results show a significant upregulation of mucin-2, zonula occludens-1, and claudin-5, and downregulation of the claudin-2 gene. Mucin-2 is an important chemical component of the mucus layer and the first-line of defense for protecting the epithelial cells in the intestinal tract from pathogens and suggested as a biomarker for intestinal health (Capaldo et al., 2017). The modulation of tight junction proteins facilitates changes in the gut permeability with occludin and claudins being major transmembrane components. These tight junction complexes together with the cytoplasmic link, zonula occludens-1 regulate the permeability of the epithelial lining (Awad et al., 2017). Claudin-1 and -5 possess the sealing properties, whereas claudin-2 is a channel forming protein. The results from this experiment show the probiotic strain increases the expression of the sealing protein genes (claudin-5 and zonula occludens-1) and downregulates the channel forming claudin-2, resulting in an increased barrier function of the epithelial lining, which will protect against translocation of bacteria from the intestinal lumen to the blood. This is supported by the finding of a lower bacterial count in the liver in the group that received the probiotic strain in the drinking water (group B).

Jejunal Morphometrics

Jejunal villi are significantly longer in group B (probiotic in drinking water) than in both the untreated control group (group C) and the Apramycin treated group (group D) (Table 1). A reciprocal picture is seen when analyzing crypt depth. Here both group C and D have

significantly deeper crypts than group B. Group C also has significantly deeper crypts than group D. It has previously been described that infection with *Salmonella* increases the depth of the crypts (Xie et al., 2020). An explanation for the increased depth may be that the cells in the crypts function as stem cells of the intestine. During restoration of the epithelia, crypt cells proliferate, differentiate, and migrate from the crypts to the villi (Garrett et al., 2010). This may explain the increased crypt depth in the control group where no probiotic was used to mitigate the inflammation. Additionally, the Apramycin treated group had significantly deeper crypts compared to other groups. The reason could be that despite the apramycin treatment, *S.* Enteritidis is causing inflammation of the epithelia and the restoration process causes the cells in the crypts to proliferate. The antibiotic does not seem to have the same mitigation of the inflammation as the probiotic since the crypts are deeper in the Apramycin treated group compared to the probiotic treated group. However, this needs further investigation.

Regardless of treatment group, confluence and shortening of villi were observed in some parts of some jejunal cross-sections. Measurements of villus width did not reveal a statistical difference between treatment groups. However, a higher number of goblet cells were apparent in the villi of group B compared to the 2 other groups, but the amount was not quantified.

Descriptive observations on the histomorphology of the cross-sections from all treatment groups revealed some degree of inflammatory cellular infiltration in the lamina propria in both the villi and beneath the crypts. These areas were dominated by mononuclear cells and in some chickens (mostly from group C and D), heterophils were also observed within the cell clusters. More severe damage to the mucosal lining was observed occasionally in some chickens (also primarily in group C and D), including epithelial sloughing at villus tips, patches of total villus destruction, and thickening of the lamina propria.

Overall, the probiotic strain improved the gut health of the chickens. Adding the probiotic (*E. faecium* 669) to the drinking water significantly reduced both the shedding of *S.* Enteritidis from the cloaca and the *S.* Enteritidis count in the ceca at d 12. Bacterial counts in the liver were significantly reduced in the group receiving probiotic in the drinking water compared to the control group. Probiotic treatment in the drinking water affected the gene expression in 4 out of the 6 investigated genes. All impacted genes were up- or downregulated in the favor of a higher epithelial integrity, which is further supported by the morphometric analysis of jejunal villi and crypts. Overall, daily probiotic treatment protected the chickens against colonization with *S.* Enteritidis and improved the intestinal integrity by upregulating the beneficial mucin-2, zonula occludens-1, and claudin-5 genes, and down regulating the pore-forming claudin-2.

DISCLOSURES

Mia Son Räfle Olsen, Ida C. N. Thøfner and Louise L. Poulsen are employed at UCPH, and Dorthe Sandvang is employed at Chr. Hansen A/S. One author is employed at Chr. Hansen A/S that produces the probiotic bacteria, which have been used in this study, resulting in a potential or perceived conflict of interest. However, the three independent academic partners (University of Copenhagen) explicitly approve that the work is at 'arm's length' from commercial interest. The first and senior author has led the process and are satisfied with the interpretation of the results.

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