Recovery of probiotic bacteria from the intestinal tract of broilers after noninvasive pre-hatch application

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Abstract

This paper reports the success of intestinal colonisation of chickens and fetuses by probiotics after different methods of pre-hatch application. Hatcheries not using *in ovo* injection of probiotics or wish to avoid the reduced hatchability associated with *in ovo* injections prefer using alternatives to *in ovo* technologies. Therefore, we used noninvasive pre-hatch application methods. This included the vertical transmission of probiotics from the mother hen to offspring, application of probiotic late in incubation and transmission of probiotics during hatch. *Enterococcus faecium* (NCIMB11181) and *Lactobacillus animalis* (DSM33570) were used as probiotics. Probiotics were applied either through drinking water for the mother hens, by dipping the eggs in a probiotic solution on days 16-18 of incubation or through drops/spray on the eggshell of the fertilised eggs. Similarly, intestinal colonisation of the probiotic in chickens was investigated either before hatch (pre-hatch) or immediately after hatch (post-hatch). Based on the performed experiments, it is concluded that *E. faecium* was vertically transmitted from the mother hen to the offspring, as *E. faecium* was recovered in 20 and 33% of the offspring pre- and post-hatch, respectively. When applied on the eggshell, the recovery of *E. faecium* before hatch depended on the application method and ranged from 0 to 9%. In contrast, *L. animalis* was not recovered before hatch. Moreover, when sampling post-hatch 100% of the chickens were colonised when *E. faecium* was used and 54% were colonised when *L. animalis* was used. Furthermore, spray application with *E. faecium* was the most successful application method as 9% of the fetuses were colonised pre-hatch and 100% became colonised post-hatch. Therefore, pre-hatch application by, for example, spray of probiotics on the eggshell can be used as an easy-to-use, noninvasive method for early life colonisation of chicken gut.

Keywords: probiotics, chickens, noninvasive application, intestinal colonisation, pre-hatch

1. Introduction

Probiotics for poultry have traditionally been added to feed or water. However, applying probiotics pre-hatch is proposed to have several advantages. Most importantly, the probiotic bacteria have better and longer-lasting effects when chickens are exposed to probiotic bacteria before intestinal microflora develops (Kubasova *et al.*, 2019). Before the establishment of the intestinal flora, the competition for adhesion sites and nutrients in the intestine is lower than in a more developed gastrointestinal microflora as found in older chickens. This means that

the probiotic bacteria have a better chance of colonising the intestine (Dominguez-Bello *et al.*, 2011; Oakley *et al.*, 2014; Patterson and Burkholder 2003; Salminen and Isolauri 2006; Stanley *et al.*, 2014). Chickens colonised by beneficial probiotic bacteria at hatch are expected to be less susceptible to pathogenic bacteria during the critical first week of life. Furthermore, when chickens hatched in industrialised hatcheries, they are not exposed to the bacterial flora from the mother hen but to bacteria originating from exploding rotten eggs, bacteria from chickens carrying a vertically transmitted infection from the mother hen and bacteria that are carried over from previous hatchings

(Kubasova *et al.*, 2019; Poulsen *et al.*, 2017; Stanley *et al.*, 2013). Therefore, applying probiotic bacteria pre-hatch is an initiative to reduce colonisation and spread of potentially pathogenic bacteria, which the newly hatched chickens are exposed to in the hatcher and on the farm. Additionally, to exert protective effects against bacterial infections, some probiotics (*Lactobacillus* spp.) have been demonstrated to have a positive effect on growth performance of broilers (Timmerman *et al.*, 2006; Zulkifli *et al.*, 2000).

Traditionally, pre-hatch administration of vaccines as well as probiotics and feed supplements (to a lesser extent) have been applied as *in ovo* injections. However, the success and safety (e.g. hatchability, and embryonic/foetal death) of this type of application highly relies on the injection site, hatchery practice and agent of interest (Peebles, 2018; Retes *et al.*, 2018). To avoid a reduction in hatchability due to *in ovo* injections, alternative pre-hatch application methods are of interest.

Therefore, this study investigates the applicability of noninvasive pre-hatch application of probiotics for early life colonisation of the chicken gut. This study aimed to specifically investigate the following:

1. whether the transmission of probiotics is possible from the mother hen to the offspring by vertical transmission;
2. whether probiotic applied on the shell of eggs late during incubation can colonise the unhatched chicks;
3. whether horizontal transmission of probiotics occurs during hatch;
4. whether colonisation before and during hatch depends on the probiotic bacteria used; and
5. whether spray application with probiotic pre-hatch is equally as effective as *in ovo* injections with probiotics when measuring colonisation on day 7 post-hatch.

2. Materials and methods

Application methods and sampling design

Two strategies for the noninvasive application of probiotics to chicken before hatch were investigated. The first strategy was to apply the probiotic indirectly to the broilers by administering the probiotic (*Enterococcus faecium*) to the drinking water of the parent birds (broiler breeders) (Figure 1A). This process will be henceforth referred to as 'vertical transmission' in the manuscript. The second strategy was to apply the probiotic directly to the fertilised eggs between days 16 and 18 of incubation. This strategy was investigated using three application methods of the probiotic onto the egg surface: (1) dipping the eggs in a probiotic solution or (2) applying in drops or (3) spraying onto the surface (Figure 1B-D). Moreover, the rate of intestinal colonisation by the probiotics before and after hatch was evaluated. Specific application methods for each strategy are described in detail in the sections below.

Figure 1. An illustration of the different application methods used in the experiments.

Probiotic strains

Lyophilised *E. faecium* (NCIMB 11181), 2×10^{11} cfu/g, and *Lactobacillus animalis* (DSMZ33570), 3×10^{10} cfu/g, were used for pre-hatch application on fertilised eggs. These strains are referred to as *E. faecium* and *L. animalis*, respectively, in the manuscript henceforth.

Application of *Enterococcus faecium* in drinking water for broiler breeders (vertical transmission) (Objective 1)

To investigate the possible vertical transmission of probiotics from the mother hen to offspring, *E. faecium* was suspended daily in drinking water for 10 broiler breeders aged 44 weeks at the onset of the trial. They had unlimited access to water, with an estimated daily water consumption of 262–328 ml water/hen/day (Aviagen, 2016, 2018). To obtain a final concentration of approximately 1×10^8 cfu/ml, resulting in an estimated average daily dose of $2.6\text{--}3.3 \times 10^{10}$ cfu/hen, 0.5 g of lyophilised *E. faecium* (NCIMB 11181) was suspended per litre tap water. The probiotic administration through drinking water to broiler breeders continued until egg collection was discontinued at the end of the trial. Eggs collected before the application of probiotics were used as negative control eggs. All eggs were stored separately at 17 °C until incubation.

Housing of broiler breeders and collection of eggs

Ten broiler breeder hens (Ross 308), 44 weeks old, and two roosters (Ross 307) were evenly distributed in two pens (approximately 8 m²/pen). Perches, nests and sand for dust bath were provided in each pen. Feed was restricted to 164 g per hen or rooster and water was applied *ad libitum*.

Eggs were laid in nests and collected daily. The eggs were collected using sterile gloves and stored in sterile egg trays without physical contact with other eggs to prevent cross-contamination between eggs.

Application of *Enterococcus faecium* and *Lactobacillus animalis* on the eggshell surface (Objectives 2-4)

The colonisation potential of probiotics applied on the eggshell of fertilised eggs were investigated in the unhatched and newly hatched chickens. For investigating the colonisation of unhatched chickens, sampling of intestine and yolk sac occurred before hatching (incubation day 19) as described in Tables 1-4, whereas for the investigation of colonisation during/after hatching, the sampling occurred during or immediately after hatching (incubation days 20-21) (Tables 1-4). Horizontal transmission of probiotic bacteria was described in experiments in which more chickens were colonised right after hatch than before hatch.

For the dipping experiment (Figure 1B), eggs were soaked for 1 s, left to dry for 1 min and soaked again for another second in an *E. faecium* solution (7×10^{10} cfu/ml) (Table S1, Experiments 3 and 4). When applying *E. faecium* as a drop

on the eggshell (Figure 1C and Table 2), 50 μ l was pipetted onto the eggshell on the side or at the blunt end of the egg after moving to the hatching trays. When the spray method was used to apply the probiotic, the amount of probiotic solution distributed per pump of the spray flask was calculated by weighing 10 pumps. Subsequently, the average of this value for calculating volumes was used with the aim of applying approximately 50 μ l/egg (Figure 1D; Tables 3 and 4). For each experiment, the cfu/ml was determined, and the volume used per egg was then used for calculating the cfu used for each egg in the experiments.

For eggshell applications, the sham-inoculation method with sterile isotonic saline by dip, drop or spray was used on control eggs as described above according to the application method in use. For every eggshell application, care was taken to minimise cooling stress on the eggs during the application procedures.

In ovo application of *Enterococcus faecium* (Objective 5)

To investigate if spray application was equally effective as *in ovo* injection of the probiotic, a comparison of the cfu in the caecum on day 7 was performed on chickens that

Table 1. Application of *Enterococcus faecium* in drinking water for broiler breeders (Objective 1).

Hatch status	Day of application	Day of sampling	Probiotic	n	cfu/egg ¹	Recovery rate of probiotic, n (%)
Unhatched	daily	19	<i>E. faecium</i>	20	3×10^{10} cfu/hen/day	4 (20)
			saline	20	— ²	0
Hatched	daily	21	<i>E. faecium</i>	33	3×10^{10} cfu/hen/day	11 (33)
			saline	20	— ²	11 (55)

¹ Exact dose of application cannot be calculated because it relied on the water intake of the hen.

² Not applicable as sampling occurred pre-hatch.

Table 2. Application of *Enterococcus faecium* as a drop on the eggshell (Objectives 2-4).

Application site	Hatch status	Day of application	Day of sampling	Probiotic	n	cfu/egg	Recovery rate of probiotic, n (%)
Drop at side of egg	unhatched	17	19	<i>E. faecium</i>	46	6×10^8	3 (7)
				saline	40	—	0
	hatched	17	20	<i>E. faecium</i>	30 ^a	3×10^8	25 (83)
				saline	40	—	0
Drop at blunt end of egg	unhatched	17	20	<i>E. faecium</i>	32 ^b	3×10^8	0 (0)
				saline	40	—	0
	hatched	17	20	<i>E. faecium</i>	14 ^c	3×10^8	6 (43)
				saline	40	—	0

^a Sampling occurred on day 20. Only fully hatched chickens were included.

^b Sampling occurred on day 20. Hatched chickens were excluded.

^c Sampling occurred on day 20. Only fully hatched chickens were included.

Table 3. Application of *Enterococcus faecium* as spray on the eggshell (Objectives 2-4).

Hatch status	Day of application	Day of sampling	Probiotic	n	cfu/egg	Recovery rate of probiotic, n (%)	Hatchability (%)
Unhatched	17	19	<i>E. faecium</i>	47	6×10 ⁸	4 (9)	–
			saline	40	–	0	–
Hatched	16	21	<i>E. faecium</i>	46	4×10 ⁸	46 (100)	94
			saline	40	–	0	93

Table 4. Application of *Lactobacillus animalis* as spray on the eggshell (Objectives 2-4).

Hatch status	Day of application	Day of sampling	Probiotic	n	cfu/egg	Recovery rate of probiotic, n (%)	Hatchability (%)
Unhatched	18	19	<i>L. animalis</i>	33	1×10 ⁷	0	–
			saline	37	–	0	–
Hatched		21	<i>L. animalis</i>	33	1×10 ⁷	18 (54)	83
			saline	29	–	0	85

received the probiotic on day 18 in the incubation period. The *in ovo* experiment was conducted at the Mississippi State University, where *E. faecium* was administered *in ovo* with Marek's vaccine (Zoetis, Parsippany, NJ, USA) in the amnion cavity (Skjøl-Rasmussen *et al.*, 2019). A selection of these samples was analysed in this study and compared with samples from the spray application (Table 5, Supplementary Table S1 – Experiment 18).

Enumeration of probiotic solutions administered

Serial 10-fold dilutions were used in the enumeration of viable bacterial counts (cfu) in all probiotic solutions. Subsequently, plating of 100 µl of each dilution on a blood agar (BA) plate prepared with 5% calf blood on BA base (Oxoid, Basingstoke, UK) for *E. faecium* and De Man, Rogosa and Sharpe's (MRS's) broth (Oxoid) for *L. animalis* was conducted. Next, agar plates were incubated aerobically (*E. faecium*) and anaerobically (*L. animalis*) at 37 °C overnight before counting. The cfu platings were conducted three times.

Fertilised eggs

For the probiotic application on the eggshell, pre-incubated fertilised eggs (Ross 308, incubation days 0-16) were obtained from a commercial hatchery in Denmark. Additionally, fertilised Layer-type eggs (LSL-Lite) from another Danish commercial hatchery were used.

Incubation and hatching

Incubation and hatching of eggs was conducted in Heka hatchers for 160 or 360 eggs (America A/S, Thisted, Denmark). Separate hatchers were used for the control and the probiotic-treated eggs. Incubation temperature was set at 37.7 °C. The humidity was set at approximately 65%, and automatic turning were conducted until 4 days before hatch, when the eggs were placed into hatching trays. Unfertilised eggs were removed from the hatchers after candling between day 10 and 14.

Table 5. Application of *Enterococcus faecium* as *in ovo* injection (Objective 5).

Hatch status	Day of application	Day of sampling	Probiotic	n	cfu/egg	Recovery rate of probiotic, n (%)	Hatchability (%)
Hatched	18	7 post-hatch	<i>E. faecium</i> with Marek's vaccine	10 ^a	1×10 ⁷	100	81

^a Ten samples were analysed in this study, and the remaining samples were analysed by Skjøl-Rasmussen *et al.* (2019).

Post-application bacteriology of unhatched and newly hatched chickens (Objectives 1-5)

Intestinal colonisation by probiotics was investigated at different time points in relation to hatching (Tables 1-5). Decapitation was then performed to euthanise newly hatched chickens and unhatched fetuses. To reduce the risk of contamination during sampling of the internal organs, every egg, euthanised foetus and chicken was dipped in 70% ethanol and allowed to dry for few minutes before sampling. Entire intestine and yolk sac were aseptically removed with sterile scissors and tweezers, after which the equipment and gloves were changed for every chicken to prevent cross-contamination. Yolk sac and intestines were used for re-isolation of *E. faecium*, both pre-hatch and immediately post-hatch. Subsequently, samples (yolk sac/intestine) were homogenised with sterile isotonic saline (1:1 ratio) until homogeneity. Next, the samples were pre-incubated aerobically at 37 °C in 30 ml of brain heart infusion (BHI) broth (Oxoid) when *E. faecium* was used as a probiotic. In contrast, when *L. animalis* was used as a probiotic, samples were incubated in MRS's broth (Oxoid) and incubated anaerobically.

After overnight incubation, a 10 µl inoculation needle was used to streak the enriched BHI broth on BA containing kanamycin (BA-kan) (1000 µg/ml) (Sigma-Aldrich, Søborg, Denmark) and incubated overnight. In the experiment where *L. animalis* was re-isolated, MRS broth was used for pre-incubation, after which samples were streaked on MRS containing kanamycin and vancomycin (200 and 300 µg/ml, respectively). Further, sub-culturing from the primary BA/MRS was performed in cases in which the yolk sac/intestinal material on the agar plate masked the analysis. In the experiment where *L. animalis* was applied five times, the yolk sac/intestinal samples were spiked with 1×10^7 cfu as positive controls.

Verification of the probiotic *Enterococcus faecium* and *Lactobacillus animalis* by strain-specific PCR

DNA was extracted following the boiling lysate method. Here, one loop (size of loop 1 µl) of the bacterial colony was suspended in 300 µl of Milli-Q water (vortex) and heated for 10 min at 100 °C. Then, the setup was centrifuged for 5 min at $14,000 \times g$ before the supernatant was transferred to a new tube and used as the DNA template.

Alternatively, DNA was extracted with the Maxwell RSC Instrument (Promega, Lystrup, Denmark) using the Maxwell RSC Cultured Cells DNA Kit (AS 1620) (Promega). Before DNA extraction, a single colony from the samples was sub-cultivated on BA (without kanamycin) to obtain a pure culture. The PCR programmes are described in the supplementary material. Strain-specific primers were developed and used for detecting probiotic bacteria by PCR (Table 6).

Table 6. Strain-specific primers used to detect *Enterococcus faecium* and *Lactobacillus animalis*.

Primer name	Sequence (5'→3')	Amplicon size (bp)
<i>E. faecium</i> _F5	AGAACAGAGAAGTAGACCAGCCA	920
<i>E. faecium</i> _R5	TGAGGCTGCGATGTTGAAAGT	
<i>L. animalis</i> _F	AGCAACTCTTCCCAGATGAACA	114
<i>L. animalis</i> _R	AGCCGGAATATAGTTAGTCGCA	

Moreover, 1% agarose gels were used to separate amplicons by electrophoresis (125 V in 40 min). Then, Generuler 1 kb plus DNA ladder (Catalogue Number SM1331; Thermo Scientific, Waltham, MA, USA) was used as a marker; bands were visualised with a Bio-Rad Molecular Imager ChemiDoc™ XRS+ (Bio-Rad, Copenhagen, Denmark) using the Image Lab™ Software (6.0 Bio-Rad).

Verification of the specific probiotic *Enterococcus faecium* strain by pulsed-field gel electrophoresis

From the experiments investigating vertical transmission of the probiotic strain from the hen to offspring, 10 random isolates were selected for verification by pulsed field gel electrophoresis. The analysis was performed as described by Jørgensen *et al.* (2017).

Investigating the presence of *Enterococcus faecium* in the salpinx of probiotic-treated broiler breeder hens

After the application of probiotic in drinking water, the egg-laying hens were euthanised. Salpinx was then removed from hens (n=8), and the mucus layer of the proximal half of the magnum and the distal part of the isthmus were scraped with a glass slide. Next, the mucus from each sample was streaked with a sterile cotton swab on BA-kan plates. Growth was described semi-quantitatively (0 = no growth, 1 = colonies in the primary streak, 2 = colonies in the second streak and 3 = colonies all the way to the third streak). Verification of the probiotic strain was conducted using the strain-specific PCR described above.

Re-isolation of the probiotic *Enterococcus faecium* from caeca of the chickens' day 4 and 7 post-hatch after application by spray and *in ovo* injection (Objective 5)

To compare how the application method affected colonisation of the chicken intestinal tract (caeca) on day 7 post-hatch *E. faecium* was applied as a spray and by *in ovo* injection. At first, 120 eggs were sprayed on day 18 of incubation with 2×10^8 cfu/egg. The newly hatched chickens (n=120) were housed in an animal facility (floor pens with wood shavings, perches and feed and water *ad libitum*)

until day 7. The control group and the probiotic treated group were housed in separate rooms to prevent cross-contamination (Supplementary Table S1 – Experiment 14). Chickens were weighed on days 1, 4 and 7. Subsequently, the presence of *E. faecium* was investigated at two time points (days 4 and 7) by euthanasia of 30 chickens from each group. Sampling was performed as described in the section ‘Post-application bacteriology of unhatched and newly hatched chickens’. Apart from that, only the caeca were used instead of the yolk sac/intestine for enumeration of the *E. faecium* probiotics. Caecal samples were stored at -20 °C until analysis. One caecum per chicken was homogenised with saline (1:1 ratio). The samples were 10-fold diluted, after which 2×10 µl was spotted on BA-kan and incubated aerobically overnight. The following day, cfu was determined. From each sample, one colony was sub-cultivated on a BA-kan and used for strain verification by PCR as described above.

The efficacy of the spray application was compared with that of *in ovo* application. Enumeration was used for this comparison of the *E. faecium* in caeca of 10 chickens (Ross 708), which received the *E. faecium* probiotic via *in ovo* injection. These chickens received a concentration of 1.4×10⁷ cfu *E. faecium*/egg into the amniotic fluid as previously described by Skjøl-Rasmussen *et al.* (2019). Their caeca were stored separately in cryotubes at -80 °C until analysis was conducted, together with the caecal samples from the spray experiment described above.

Ethics

The animal experiments were conducted in agreement with the Danish animal expectorate under the licence number 2019-15-0201-01619.

Statistics

The results are presented as rates (e.g. hatchability and re-isolation of probiotic) and means with standard deviation (SD) (cfu and body weight). For normalising cfu counts, log₁₀ transformation was applied. All statistical analysis was performed using Prism 8 for Windows (version 8.4.0, GraphPad Software, Inc., La Jolla, CA, USA). The Chi-square test was used for comparing colonisation rates of the pre- and post-hatch intestines; Fisher’s exact test was used to compare differences in hatchability. In addition, one way ANOVA followed by Tukey’s multiple comparison test was used to calculate differences in the body weight on days 1, 4 and 7 and for comparing cfu values on days 4 and 7 after application by spray and *in ovo* injection, respectively. Significance levels were set to 0.05.

3. Results

The results from different application experiments are summarised in Tables 1-5. Results concerning the specific study objectives 1-5 are described in detail in the sections below.

Vertical transmission of *E. faecium* from breeders to broilers and the presence of *E. faecium* in the salpinx of the probiotic-treated breeders

The probiotic *E. faecium* passed from the mother hen to the offspring (Table 1). In the unhatched chickens, the probiotic strain was recovered from 20% of the chicks at the time of hatch, whereas 33% of the chicks had the probiotic strain in their intestine/yolk sac. These results are illustrated as the first bars in Figure 2 (‘Vertical’). Although the incubation of eggs occurred in different hatchers, 55% of the untreated control chickens were colonised at hatch. The two hatchers were then placed in the same room, after which the hatcher used for the control eggs was subsequently found to be contaminated with the probiotic strain. After sampling, an open petri dish with BHI was placed in the hatcher for 4 h. Next, 100 µl of the broth was spread on a BA-kan plate and incubated overnight at 37 °C. Furthermore, it was discovered that the ventilation outlet in the hatcher was unfiltered. Therefore, control chickens were colonised during hatch in the control hatcher. Subsequently, the hatchers were disinfected and the control hatcher was placed in a separate room for future experiments.

From the investigation of the broiler breeder hens, the probiotic strain was found to be present in the proximal part of the magnum of the salpinx in four of the eight hens by direct plating. The growth varied from limited (score 1) in one hen, moderate growth (score 2) in two hens and extensive growth (score 3) in one hen. In three of these four hens, the probiotic strain was found in the distal part of isthmus, one with limited growth (score 1) and two with extensive growth (score 3). In total, four of the eight had a salpinx that was positive for growth at one sampling site (n=1) or two sampling sites (n=3). Strain identity was confirmed by strain-specific PCR.

Investigating colonisation of chickens pre-hatch

When using *E. faecium* and sampling pre-hatch, none of the chickens were colonised after applying a drop at the blunt end (0/32). However, when applying the drop at the side of the eggs, 7% (3/46) were colonised; when applying the probiotic as a spray on the side of the eggs, 9% (4/47) of the yolk sacs/intestines became colonised. The highest pre-hatch colonisation rate was noted when the probiotic was applied in the drinking water for the mother hen in which 20% (4/20) of the offspring were colonised pre-hatch (Table 1). Moreover, in the experiment in which the eggs

Figure 2. Percentage of chickens colonised pre-hatch (A) and post-hatch (B) with different application methods using *Enterococcus faecium* as probiotic.

were dipped twice in a concentration of 7×10^{10} cfu/ml, the re-isolation rate was 57% pre-hatch. At hatch, 100% were colonised; however, only 30% of the dipped chickens hatched (Supplementary Table S1 – Experiment 3 and 4).

The anatomy of the embryonated egg is illustrated in Figure 1. As shown, no blood vessels are present at the blunt end where the air cell is found. At the side of the egg, there is a higher probability that the probiotic bacteria came in close contact with blood vessels in either the vitelline or allantoic membranes, which facilitated the colonisation of the foetal intestine.

In contrast, when the probiotic was administered as a spray at the side of the eggs, the probiotic recovery was 9% in the unhatched foetuses. However, the *L. animalis*, which was applied as a spray (Table 3), was not re-isolated from any unhatched foetuses (0/33).

Investigating colonisation of chickens post-hatch

When sampling chickens immediately after hatch, the rate of re-isolation of the probiotic strain varied according to the application method (Figure 2B). However, with vertical application of the probiotic to mother hen, 33% of the newly hatched chickens were colonised (Table 1, Figure 2B).

In the experiments in which a drop was placed at the side or the blunt end of the egg (Table 2), the re-isolation rates were 83 and 43%, respectively. Similarly, in the experiments where spray was used as an application method, the re-isolation was 100% when treated with *E. faecium* (Table 3) and 54% in the group treated with *L. animalis* (Table 4).

Investigating caecal colonisation of 4- and 7-day old chickens after spray application and *in ovo* injection on day 18 of incubation

The probiotic *E. faecium* strain was re-isolated from the caecum on days 4 and 7 concentrations stated in Figure 3. Control group chickens on days 4 ($n=30$) and 7 ($n=30$) were negative for the probiotic *E. faecium*. However, in the chickens originating from the sprayed eggs, the mean log (cfu) of the probiotic strain was significantly lower in the caeca of chickens on day 4 (mean log (cfu) $\pm$ SD: 6.27 ± 1.37) than on day 7 ($P=0.0007$). Therefore, the chickens, which received the probiotic as an injection into the amniotic fluid, had a mean log (cfu) of 6.05 ± 0.66 .

Hatchability

The hatchability did not differ after spray application on Ross 308 eggs with sterile media (placebo) or *E. faecium*. The hatchability varied from 93 to 94% (Table 3) ($P>0.9999$). Likewise, no difference was observed on the hatchability after application of *L. animalis* in LSL-Lite eggs ($P=0.7379$), resulting in hatchabilities on 83 and 85% in the treated and control groups, respectively (Table 4). However, in the *in ovo* injection trial, the control group was injected with Marek's vaccine alone, and hatchability was compared with that of the group injected with Marek's vaccine and the *E. faecium* probiotic. Based on the results, no significant difference in hatchability was found between these groups (Skjøt-Rasmussen *et al.*, 2019). Only in the experiment in which eggs were dipped in the probiotic solution did the hatchability differ significantly between the control and treated groups (Supplementary Table S1 – Experiments 3 and 4).

Figure 3. The mean log (cfu) of *Enterococcus faecium* per gram caeca in chickens on days 4 and 7 after spray application on the eggshell and *in ovo* injection (only day 7), with *E. faecium* probiotic on day 18 of incubation (Mean and standard deviation are shown).

The hatchability was 30% in the probiotic group, in which eggs were dipped in 7×10^{10} cfu/ml *E. faecium* compared with that of the control group, in which eggs were dipped in saline. Furthermore, the unhatched chickens in the probiotic group (dip application) were found to be dead late in the incubation period. All dead chickens were investigated for pathogens as *E. coli* or *E. faecalis* in the yolk sac of the chickens. Similarly, hatchability after *in ovo* injection was 81% in both the probiotic and control groups. This investigation was performed by sampling the yolk sac with a sterile cotton swab, streaking it on a BA and incubating aerobically overnight at 37 °C. None of the samples showed any bacterial growth. However, it was noticed that the probiotic solution used for dipping covered the eggs as a film after drying, thus leading to suffocation of the foetuses owing to the blocking of the pores in the eggshell.

Body weight of chickens receiving *Enterococcus faecium* as a spray pre-hatch

The mean body weight of chickens on days 1, 4 and 7 were 45.47 ± 3.54 and 44.80 ± 3.49 g ($P=0.3014$), 90.77 ± 11.56 and 92.10 ± 10.06 g ($P=0.6355$) and 139.6 ± 22.57 and 144.0 ± 18.60 g ($P=0.6255$) in the control and probiotic-treated groups, respectively (Figure 4). However, no statistical difference in the body weight was observed between the groups within the three sampling days.

Figure 4. Mean bodyweight of the control ($n=30$) and probiotic-treated chicks (*E. faecium*) ($n=30$) on days 4 and 7. No statistical difference was found between the control and probiotic-treated groups on days 1, 4 and 7 (Experiment 14) (Mean and standard deviation is shown).

4. Discussion

This study demonstrated that the probiotic *E. faecium* was transferred from the hen through the egg to the offspring by daily oral application of the probiotic to the hen *ad libitum* (Objective 1). Before and right after hatch, 20 and 33% of the offspring were colonised, respectively. The higher percentage colonised at hatch indicated that the probiotic was spread horizontally in the hatcher during hatch (Objective 2). Such transmission has previously been described for *Escherichia coli* and *Enterococcus faecalis*, which is also proposed to be transmitted between chickens during hatch (Olsen *et al.*, 2012; Poulsen *et al.*, 2017). In terms of protection against bacterial pathogens in the first week of life, the ideal probiotic should be present, and colonising the gut of the chickens before the chicken is exposed to the pathogens is important. Thus, colonisation at the time of leaving the eggshell is proposed to be optimal timing. Moreover, an explanation for the relatively low colonisation rate (33%) is proposed to be that the concentration of the probiotic was low on/in the eggs, which received the bacteria from the mother hen. However, neither the concentration on the eggshells nor that on the hen gut was investigated, thus needing further research.

Furthermore, how long the colonisation time lasts after finalising the application of probiotic in drinking water was not assessed. Similarly, whether the colonisation of 33% of the chickens exerted a protective effect against the horizontal transmission of pathogens in the hatcher remains to be investigated. Therefore, the long-term effect of vertical transmission of probiotics on broiler performance needs further investigation as well. The probiotic was re-isolated from the salpinx of 50% of the investigated breeder hens, proposing that the bacteria ascended from the cloacal region into the oviduct/salpinx. The shell gland mucosa everts into the cloaca during ovipositioning and is thus exposed to the cloacal environment every time an egg is laid. In adult egg-laying birds, the major route of transmission in oviduct infections is proposed to be bacteria ascending from the gut or environment through the cloaca (Landman *et al.*, 2013; Pors *et al.*, 2014). However, the consequences/benefits of the presence of nonpathogenic bacteria in the oviduct remains unclarified. Additionally, it is unclear whether a distinct microbiome for the healthy egg-producing oviduct is present. Therefore, despite the small number of hens investigated, the presence of the probiotic in the oviduct (magnum and isthmus) suggests that a true vertical transmission into the egg occurs during egg formation. However, eggshell deposition of probiotic from the cloaca cannot be excluded either.

The ability of the probiotic strain to colonise the unhatched chickens when administered onto the eggshell on days 16–18 of incubation varied from 0 to 9% (Objective 2). The lowest colonisation was found when probiotic *E. faecium* was administered in drops on the blunt end of the egg. However, the highest colonisation was found when the probiotic was administered as a spray (9%). Furthermore, as described above, placing the drop at the blunt end did not result in colonisation of any chicken before hatch, whereas 7% were colonised when the drop was placed at the side of the egg. This result indicates that the *E. faecium* is transported by blood vessels present at the side of the egg but does not reach the blunt end where the air cell is present (Figure 1). The application by drops on the side of the eggs resulted in a lower colonisation rate after hatch than when spray was used to administer probiotic. This result accounts for the fact that the sampling occurred one day earlier and therefore, less time was allowed for horizontal transmission in the hatcher (Objective 3). Another explanation is that by drop application, only a limited area of the eggshell was exposed to the probiotic than the spray, where approximately 1/3 to 1/2 of the area of the shell is exposed. These hypotheses need verification by further investigations.

Additionally, when *L. animalis* was used as a probiotic, no pre-hatch colonisation occurred (Objective 4). Preliminary unpublished data showed that *L. animalis* survived for 4 days at 37 °C under aerobic conditions at the shell of egg.

Therefore, the lack of colonisation is proposed to be owing to a lower ability to pass through the eggshell barriers and reach the foetus. In the newly hatched chickens sprayed with *L. animalis*, the bacteria could be re-isolated from 54% of the chickens (Objective 3 and 4).

Therefore, the results indicate that considerable colonisation occurs during hatch, resulting in a 2.8- to 11.5-fold increase in *E. faecium* and unmeasurable for *L. animalis*. At the time of hatch, chickens from the probiotic-treated eggs will be colonised when they start eating whatever soft and edible is present in the hatcher (e.g. shell membranes, meconium and egg content from exploded eggs) (Objective 3). Thus, the probiotic strain exerted protection by competitive exclusion, pathogen inhibition (De Oliveira *et al.*, 2014; Varmuzova *et al.*, 2016) and enhancement of gut integrity. However, during hatch, the chickens were colonised or exposed to pathogens, such as *E. coli* and *E. faecalis* together with the probiotic. The question of how important it is to be colonised by bacteria exerting a positive effect on gut health before exposure to pathogens requires more investigations.

The noninvasive application of probiotics did not affect the hatchability except for one experiment where eggs were dipped in a concentrated solution of *E. faecium*. The difference in hatchability among the experiments described in Tables 3, 4 and 5 most reflects that the eggs were provided by different hatcheries with different management procedures for storage, candling, sorting and so on of eggs.

The presence of the probiotic strain of *E. faecium* for 4 and 7 days after hatch at relatively high concentrations at both time points indicates that the bacteria survived. It shows that the bacteria colonised the intestinal tract of the newly hatched chicken for at least the first week of life (Objective 5). Comparison with *in ovo* injected birds was considered an application control for the spray application because *in ovo* injection makes it easier to control the concentration in all eggs (Objective 5). The caecal content of the probiotic was significantly higher in the caecal sacs of the chickens which received the probiotic as a spray on the eggshell, than in those which received the probiotic as *in ovo* injection. The cfu in the spray solution was 10 times higher than the cfu in the injected solution (10^8 vs 10^7 cfu/egg). Furthermore, the management of the two groups of treatments differed which makes it difficult to draw definite conclusions.

5. Conclusions

E. faecium was successfully recovered after vertical transmission from mother hen to offspring (Objective 1). The above described experiments showed that noninvasive application of an *E. faecium* probiotic was a successful method to colonise a proportion of the foetuses before hatch (Objectives 1 and 2). However, not all probiotic strains were equally successful (Objective 4).

Furthermore, it can be concluded that the probiotics were horizontally transmitted during hatch and a high percentage of chickens were colonised at hatch. Moreover, the proportion of colonised chickens depended on the application method and the probiotic used (Objective 3).

Therefore, we propose that spraying the probiotics on fertilised eggs should be used as an 'easy-to-use' method for the noninvasive mass application of probiotics for both small and large hatcheries in the future (Objective 5).

Finally, alternative methods for the application of probiotics will be an advantage in broiler production as an alternative method to address the reduced hatchability when *in ovo* injection application of probiotics is used. It should extend to the use of probiotics to hatcheries which are not using the *in ovo* technology. Furthermore, this alternative, easy-to-use and relatively low-tech methods prove valuable for small scale/non-automated hatcheries and fancy breed breeders.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0227>.

Table S1. Summary of all application experiments.

Table S2. Preparation of mastermix for M74_F5/R5 (*Enterococcus faecium*, NCIMB11181) and LA51_F/R (*Lactobacillus animalis*, DSM33570).

Table S3. PCR programs for *Enterococcus faecium* (NCIMB11181) and *Lactobacillus animalis* (DSM33570).

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Conflicts of interest

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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 is satisfied that the results are independent.

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