1 **Evaluation of a new probiotic concept for broilers**

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15 **Primary audience:** plant managers, veterinarians, nutritionists

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SUMMARY

Probiotics were introduced as a spray directly in the hatcher when chickens started to leave the eggs which potentially could reduce the horizontal transmission and colonization with pathogenic bacteria. The single introduction of probiotics could limit the cost compared to multiple introductions with feed and/or water. A mixture of five probiotic strains belonging to *Escherichia coli*, *Enterococcus faecalis*, *Lactobacillus agilis* and *Lactobacillus rhamnosus* was tested with two independent flocks of broilers (Ross 308). For each experiment, a comparison was made to an untreated control flock on the same farm. At day 14 of production the probiotic strains were re-isolated from ileum of euthanized chickens. The first week mortality was slightly increased in the probiotic flock (0.42%) compared to the control (0.35%) in experiment 1, however, it was higher in the control flock (1.45%) compared to the probiotic flock (1.12%) in experiment 2. The average weight of chickens that could be slaughtered for consumption was increased by 3.5% in the probiotic flocks compared to the control flocks, resulting in a 1.9% higher total weight of slaughtered chickens in the probiotics treated flocks compared to the control as a mean of the two experiments. The number of condemned animals was within the normal range for the production system and could not directly be related to effects of probiotics. Although one probiotic strain of *E. coli* was isolated from dead animals, the probiotics did not affect the proportion of chickens which died due to *E. coli* during the first week compared to the control.

DESCRIPTION OF PROBLEM

Bacterial infections in industrialized chickens can cause high mortality and major economic costs [1, 2]. Bacteria including *Escherichia coli*, enterococci and staphylococci have been reported most frequently to contribute to bacterial disease in poultry, being associated with septicemia, omphalitis, salpingitis and endocarditis [3, 4, 5]. Vertical transmission from the parent hen to the chicks and subsequent horizontal transmission in the hatcher has been considered to be the main route of the infectious agent to spread [5, 6].

The chicken's first week of live has been found critical and the main bacterial challenge has been *E. coli* [7]. Producers aim to keep first week mortality (FWM) below 0.7% [8]. Antibiotics have traditionally been preferred to control FWM. However, due to the

47 development of antibiotic-resistant bacteria, alternative treatment methods have been proposed to
48 reduce bacterial infections.

49 Newly hatched chicks in industrialized settings can be exposed to the microflora
50 found on the eggshells in the hatcheries and bacteria of chicks which may hatch with a vertical
51 transmitted infection [5]. The colonization route of chickens provided the idea to use a new way
52 to deliver probiotics to the chickens. A novel application method (new probiotic concept, **NPC**)
53 has been introduced applying the probiotic bacteria in the hatcheries during the hatching process.
54 The probiotics were sprayed in the hatchery at the time point where 50% of the eggs had hatched.
55 The method relied on the further horizontal transmission of the probiotic strains and colonization
56 of the hatching chicks.

57 Probiotics have been defined as “a live microorganisms which, when administered
58 in adequate amounts, confer a health benefit on the host” [9]. Probiotics have been used as
59 prophylactic treatment of industrialized chickens and a wide range of bacterial strains and
60 application methods have been described [10-12]. Several bacterial species have been
61 investigated for a probiotic potential. Most significantly lactobacilli have been shown to have a
62 protective effect against pathogenic bacteria as well as a beneficial effect on the digestive and
63 immune responses in chickens. Additionally, both *Enterococcus faecium* and *Escherichia coli*
64 have been shown to have a similar positive effect in chickens under experimental conditions [13-
65 19].

66 The application of probiotics in broiler production has been well studied and
67 probiotics in particular have been demonstrated to have positive effects in the control of
68 pathogenic bacteria, including species of *Campylobacter*, *Salmonella* as well as *E. coli* [11, 20,
69 21]. Unfortunately most studies performed and positive results reported have been obtained
70 under experimental conditions and not on farm level.

71 The objective of this study was to investigate NPC in an industrialized production
72 setting and to test the effect of the NPC on hatchability, FWM, feed conversion ratio (**FCR**),
73 production efficiency factor (**PEF**) and total slaughtered weight.

74

75 MATERIALS AND METHODS

76 *Approval of the animal experiment*

77 The experiment was approved by The Danish Veterinary and Food Administration,
78 the Ministry of Environment and Food (case no. 2015-29-21-00714).

79

80 *Probiotic strains*

81 Strains listed in Table 1 were provided freeze-dried in a powder formulation.
82 Immediately before spraying, the strains were solubilized in deionized water. Whole genome
83 sequencing (**WGS**) was used to characterize the strains genetically and to design strain specific
84 PCR primers for identification [22].

85

86 *Distribution of probiotics directly in the hatcher*

87 The hatchers were of model Petersime (Zulte, Belgium) [25]. The spray devise to
88 deliver the probiotic spray was designed as an independent unit that was placed in the hatcher at
89 the same time that it was loaded with fertile eggs [26]. A dose of probiotics was approximately
90 5×10^8 cell forming units (**CFU**) for each chick and was delivered in 1.0 ml deionized water.
91 After spraying, a sample of the probiotic solution was collected for counting of CFU (Table 1).

92

93 *Farm*

94 The NPC was tested in a conventional Danish broiler production farm.
95 Experiments 1 and 2 were carried out on the same farm in two similar buildings with respect to
96 size, feed, watering system, ventilation and temperature controls. In the first experiment 15,400
97 Ross 308 chicks were inserted in both the probiotic treated and the control group. In the second
98 experiment 15,000 chicks were included each group (Table 2). In experiment 1, the probiotic
99 treated chicks were inserted in house 1, and the control chicks in house 2. In the second
100 experiment, chicks treated with probiotics were inserted in house 2 and house 1 left for the

control flock. The farm performed a thinning after 34 days where 5,000 and 4,500 broilers were removed for slaughter from each house, respectively from the two experiments followed by a final delivery to slaughter at day 38. The effect of the probiotics was measured by FCR [27], FWM and PEF [27], as well as total and average weight of birds at slaughter.

At day 14, 25 birds from the treatment and control flocks were euthanized by cervical dislocation and contents of ileum (n=25) and cecum (n=25) were collected and tested for the presense of the probiotic strains by culturing and PCR. Dead chickens were collected throughout the production period and immediately stored at – 20 °C.

Probiotic strain recovery and identification

The content of the ileum from the 25 euthanized birds (approximately 1 g wet weight per bird) were frozen in glycerol (15% vol/vol final conc.) and stored at - 80 °C until further analysis. Samples were plated on Man de Rogosa, Sharpe broth [29] for recovery of lactobacilli and blood agar plates (**BA**) (5% calf blood in blood agar base [30] for recovery of *Enterococcus faecalis* and *E. coli* and incubated overnight at 37 °C. From all BA and MRS agar plates, five presumptive *E. coli*, *E. faecalis* and *L. agilis* colonies were isolated per plate and pooled before suspension in in 100 µL sterile filtered ultraclean water [31] for preparation of DNA templates. The samples were boiled for 10 min and centrifuged at 14,000 ×g for 5 minutes. The supernatant was used as DNA template for PCR. PCR identification of the probiotic samples was made, using strain-specific primers (Table 2). To confirm the presence of the probiotic *E. faecalis* and *L. agilis* strain, boiling lysates were prepared in 85 µL sterile filtered ultraclean water with 5 µl lysozyme (10 µg/ml) and 10 µl lysis buffer (200 mM Tris HCl, 500 mM KCl, pH 8.4) at 37 °C for 20 min. Hereafter, 4 µl protein kinase K was added and digested for 60 min at 56 °C. The samples were centrifuged at 15,000 ×g for 5 min at 5 °C, the supernatant was transferred to a new tube and used for DNA PCR template. For samples found positive by PCR, single colonies from the primary plates were confirmed positive by the strain-specific PCR tests. Five single colonies were used to prepare DNA template from each sample.

Post mortem examination of dead chickens

130 The birds collected underwent a full *post mortem* examination including
131 bacteriological sampling upon indication when vascular disturbances, exudations or enlargement
132 of the liver was present. The yolk sac and/or liver were sampled with a sterile cotton swab after
133 sterilising the tissue with a hot burning iron

134

135 ***Bacteriology of E. coli and E. faecalis isolates collected during post mortem examination***

136 Bacteriological samples collected during *post mortem* examinations were plated on
137 BA and grown aerobically overnight at 37 °C. From plates showing abundant and pure growth of
138 presumptive *E. coli* colonies (circular with entire margins, low convex, medium size, non-
139 transparent with a light greyish colour on BA) or *E. faecalis* colonies (smooth, circular colonies
140 with entire margins, low convex and small size with a white to greyish colour), one single colony
141 per sample was sub-cultured in Brain Heart Infusion Broth (**BHI**) [32] and stored in 15%
142 glycerol at -80°C. All isolates were tested with the strain-specific PCRs to verify whether the
143 isolates were the *E. coli* and *E. faecalis* used as probiotics. In addition, all isolates which tested
144 positive in the strain-specific PCR were typed by Pulsed-Field-Gel-Electrophoresis (PFGE) for
145 comparison to the probiotic *E. coli* and *E. faecalis* (Table 1). The PFGE protocol for *E. coli* was
146 used in a modified version [33]. The restriction enzyme *XbaI* was used [34]. The PFGE protocol
147 for *E. faecalis* was described by Jørgensen et al. [35].

148

149 ***Statistical methods***

150 Fischer's exact test was used for the analysis of data from GraphPad Prism5 [36] with a level of
151 significance of 95%.

152 RESULTS AND DISCUSSION

153 *Delivery of spray in the hatcher*

154 The spray device and delivery of the spray was initially tested without eggs in a
155 hatcher with paper in the trays. Deionized water with Avibblue [37] as trace dye was used. Over
156 50% of the trays received the dye after a full spray event. Aerosols will also be taken up by the
157 chicks and a higher proportion than 50% of chickens will probably receive the probiotics with a
158 full hatcher.

159 The hatchability was not affected by the NPC treatment. The humidity increased
160 from 29.3 to 30.8% during the 7 s activation of the spray device, however, the spray device did
161 not affect the automatic humidity control in the hatcher.

162

163 *Survival of probiotic bacteria in the hatcher*

164 During the 24 h that the probiotic cultures were left in the hatcher at 37 °C, the
165 CFU decreased with approximately one order of magnitude (Table 1).

166

167 *FWM and post mortem examination*

168 In experiment 1, the FWM was 0.42 and 0.35% in the treated and the control
169 house, respectively. With FWM below 0.7%, the two flocks can be regarded as normal
170 performing in regard of FWM [8]. In the second experiment FWM was 1.45 and 1.12% in the
171 control and treated house, respectively. For this experiment the FWM may be regarded as higher
172 than expected according to the broiler management manual [8]. However, the broiler breeders of
173 the flock in experiment 2 were 54 weeks compared to 37 weeks in the first experiment which
174 could explain the higher mortality. A total of 233 chickens dead on their own were collected for
175 *post mortem* and bacteriological analysis. The most prominent lesions for chicken that died
176 during the first week were yolk sac infections, sepsis, peritonitis, pericarditis and perihepatitis.
177 For the first experiment, only 26 and 16% of the chickens collected as dead on their own during
178 the first week from the probiotics and control flock, respectively allowed the isolation of *E. coli*
179 in pure growth which could indicate an effect of the probiotic treatment, however, for the second

180 experiment the similar figures were 73 and 95% for the probiotics and control flock, respectively
181 [38] indicating that the probiotics did not affect the proportion of chickens which died due to *E.*
182 *coli* during the first week.

183 The strains isolated during *post mortem* analysis were investigated with the strain
184 specific PCR methods developed for the probiotic strains and were furthermore investigated by
185 PFGE. In Experiment 1, none of the *E. coli* isolated from diseased chickens of the control group
186 were identified as the probiotic *E. coli* strain by the strain-specific PCR. However, four of the *E.*
187 *coli* isolated from the probiotic treated flock were positive for the strain-specific PCR of strain
188 730-281114-10n and showed identical PFGE banding patterns to the strain delivered in the
189 inoculum. Three isolates were obtained from yolk sac infections and the fourth from sepsis.
190 Furthermore, the two *E. faecalis* strains isolated tested positive in the strain-specific PCR for
191 probiotic *E. faecalis* strain 336 and showed identical banding patterns by PFGE analysis
192 compared to the probiotic strain. The two isolates were obtained from yolk sac infection and
193 sepsis, respectively. The probiotic *E. coli* strain 730-281114-10n and *E. faecalis* strain 336 were
194 excluded from experiment 2 based on the negative results.

195

196 ***Re-isolation of probiotic bacteria from 14 day old chicks***

197 In experiment 1, the probiotic *E. faecalis* strain 336 and *E. coli* strain 730-281114-
198 10n were identified by PCR in 4 and 8% of collected ileum samples, respectively. The probiotic
199 *L. agilis* strain 268a was identified by PCR in 20 and 60% of the collected cecum and ileum
200 samples, respectively.

201 In experiment 2, the probiotic *E. coli* strain L12 was identified by PCR in 8% of
202 the collected samples, while *L. agilis* strain 268a was identified by PCR in 10 and 35% of the
203 collected cecum and ileum samples, respectively. Two isolates of *L. agilis* from ileum were
204 subsequently analysed by WGS and SNP analysis to confirm the identity to strain 268a of *L.*
205 *agilis*. The two sequences only differed from the 268a genome by a single SNP and the isolates
206 were concluded to be identical to the original strain.

207 In previous studies performed in experimental animal facilities it was demonstrated
208 that the probiotic bacteria used in these two field experiments could be re-isolated from the

majority of the treated chickens (data not shown). The intestinal flora will probably not change dramatically with time and the probiotic strains may be transient bacteria colonizing the chickens for the first weeks. In the current study the probiotic strain added with NPC were present during the first period of the production period where the chickens show the highest susceptibility to infections by pathogenic bacteria. The commercial strain SP1 was not tested for survival in the current study but has, previously been shown to survive for 30 days at 4°C in emmer beverage [39].

Total weight and condemnation at slaughter

The total mortality included dead animals in production, animals that died during transport and condemned animals. The total mortality was significantly higher in the probiotic flocks compared to the control flocks in both experiments (Table 3). The higher mortality in the probiotic treated flocks was balanced out by an increased in mean weight in the probiotic flocks. The mean weight of chickens accepted for consumption was 3.3 and 3.7% higher in the probiotic flocks compared to the control in experiment 1 and 2, respectively. The final slaughtered weight accepted for consumption was higher in the probiotic groups compared to the control flocks in both experiments by 1.7 and 2.1% for experiment 1 and 2, respectively (Table 3).

The number of condemned animals was higher in the probiotic treated flock (1.66%) compared to the control flock (1.49%) in the first experiment, while the number of condemned animals was higher in the control flock (1.02%) compared to the probiotic flock (0.93%) in the second experiment, neither were significant (Table 4).

The number of condemned animals was significantly higher in the first experiment compared to the second in both the probiotic houses and the control houses ($p < 0.001$). The proportion of condemned animals in the two houses were comparable to the three additional houses of the farm (data not shown) and to the normal in Danish production [40]. The higher condemnation in the first experiment compared to the second was mainly due to a significantly higher number chickens condemned due to irregular color/smell/consistency ($p < 0.001$) (Table 4). This type of condemnation could not be directly related to infectious disease and was therefore not considered to have any association with the NPC treatment. Condemnations related to ascites were higher for the probiotics treatments compared to controls for both experiments,

239 however, this condition predominally has been associated with non-infectious causes. The
240 number of animals condemned due to skin discoloration was not consistently related to the
241 treatment with probiotics.

242

243 *FCR and PEF*

244 The PEF [28] index includes body weight, mortality and FCR [28] and can be used
245 to evaluate the efficacy of the broiler productionon where a high value indicates an effective
246 production. At the day of slaughter in the first experiment, the PEFs were 351 and 365 for the
247 control flock and probiotic treated flock, respectively (Table 5). In the second experiment the
248 PEFs were 379 and 375 for the control and probiotic treated flocks, respectively. However, PEF
249 values include weight, mortality and FCR of the animals sent to the slaughter facilities. When
250 calculating the adjusted FCR and adjusted PEF for the animals accepted for consumption, the
251 FCR per accepted animal was poorer and the adjusted PEF was overall lower for all groups
252 compared to the control. However, the adjusted PEF was higher in the probiotic flocks in both
253 experiments (Table 5).

254

255 CONCLUSIONS AND APPLICATIONS

- 256 1. The test of the new probiotic concept under production conditions showed that the
257 probiotic bacteria sprayed in the hatcher during the hatching process did not harm the
258 chicks. However, in the first experiment, one probiotic strain of *E. coli* and one of *E.*
259 *faecalis* was isolated from dead chicken in production and these strains were not included
260 in the second experiment.
- 261 2. A higher total mortality was observed in the probiotic treatments compared to the control
262 and the reasons need further examination. However, FWM including *E. coli* related
263 infections or condemnations were not higher in the probiotics treated flocks compared to
264 the control.
- 265 3. The mean weight of chickens and the total slaughtered weight accepted for consumption
266 was higher in the probiotic flocks compared to the control in both experiments.

- 267 4. The higher weight of animals in the probiotic treatment compared to the control was
268 reflected in a higher PEF in the probiotic treated flocks compared to the control flocks
269 but only when animals available for consumption were considered in the calculations.
270 5. Further investigations with the new probiotic concept are suggested to be done to test the
271 persistence of the probiotics in the flocks and to test the concept under different
272 management conditions.

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360 Purification Kit as recommended by the producer. DNA was sequenced by 2 × 251 bp paired-end
361 sequencing on a MiSeq instrument (Illumina) and short reads were assembled using SPAdes
362 v3.5.0 [23]. The Single Nucleotide Polymorphism (SNP) analysis was performed using the CSI
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375 25. The inner size of the egg trays was 37 cm wide and 57 cm long and could hold 100 eggs. The
376 sides of the trays were perforated vertically by 12 mm spacing leaving bars of 3 mm which
377 allowed ventilation as well as the passage of spray with probiotics. The two sides of the hatcher
378 each had room for 192 egg trays (12 stacks of 16 cm in height) mounted on 3 wagons. For the
379 experiments, only one side of the hatcher was treated with probiotics.

380

381 26. The spray device was based on a motorized knapsack sprayer (Maruyama, model 2
382 MSB151DX, ME International Installation GmbH, Achim, Germany) that provided 2 l spray per
383 min. The unit was installed with a wireless switch (Keeloq, RF Solutions Ltd, Lewes, UK) and
384 timer (TMR 48, Crouzet, Valence France) and it was powered by a rechargeable battery. Eight
385 nozzles were mounted on the side wall of the hatcher and they were connected to the tank and
386 pump by flexible tubing. The nozzles were of type Hcx 4 Gallon Blue (CBP2793, Micron Group,
387 Bromyard). The device was activated by the wireless switch at 24 h before the hatcher was
388 emptied. The device delivered 7 s pulses of spray with intervals of 7 min. It took 8 h to empty
389 the 13 l of the tank. The movement of chicks and contact in boxes allowed further horizontal
390 spread of probiotics between the chicks. The spray equipment including nozzles was cleaned
391 with deionized water after application and externally disinfected with 70% ethanol.

392

393 27. The FCR was measured to describe the efficiency of the chicken to convert feed into body
394 mass and was calculated as described by Aviagen in Ross Broiler Management Handbook [28].
395 The PEF is a performance measuring tool developed to give an overall performance value,
396 giving a standardized value for technical performance, including weight increase and mortality
397 [28].

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Table 1. Probiotic strains and doses of cell forming units (CFU) used in the two experiments.

			Dry formulation of probiotics per treatment	Predicted dose	Measured dose in tank when the hatcher was emptied
Species	Strain	Experiment	g	CFU/ml	CFU/ml
<i>E. coli</i>	730-281114-10n	1	20	3.2×10^7	5.6×10^6
<i>E. coli</i>	L12	2	20	1.3×10^6	ND ¹
<i>E. faecalis</i>	336	1	20	3.7×10^5	7.6×10^7
<i>L. agilis</i>	268a	1	20	2.9×10^8	2.2×10^7
		2	20	2.9×10^8	2.2×10^7
<i>L. rhamnosus</i>	SACCO SP1	1	10	2.1×10^8	ND
		2	10	2.1×10^8	ND
ND ¹ , not detected					

Table 2. Genomic accession numbers of strains, PCR primers and PCR conditions used to detect probiotic strains.

Species	Strain	Accession numbers of genomic sequences	Primers 5'-3'	Annealing temp. [C °]	Product size [bp ¹]	Prediction of match to template
<i>E. coli</i>	730-281114-10n	PKGL01000000	F- GCATTTGCGTTAAGCAGTCA R- ACAACGAAGCACTTCCCAAC	58	140	Match target and 26 other <i>E. coli</i> in NCBI ²
<i>E. coli</i>	L12	PKGJ01000000	F- GGTCAGGGGAACAGCATAAC R- GGATAAACCGGCTCATTTC	58	200	Match target and >100 hits to other <i>E. coli</i> in NCBI
<i>E. faecalis</i>	336	PKGJ01000000	F- AGAGCAAACGGCTCTCCTTA R- TTTACGCTTTGGGTTTACGG	58	957	Match only to target in NCBI
<i>L. agilis</i>	268a	PKGJ01000000	F- ATCCCAACTACGGGCTAACG R- CCTCAAAATGGTCAAGAATTAGC	58	322	Match target and PKGM01000005, PKGM01000047 of <i>L. agilis</i> strain 273 in NCBI

¹ bp, base pairs

² NCBI, National Center for Biotechnology Information

1 Table 3. Production parameters for experiment 1 and 2.

Animals	Experiment 1			Experiment 2		
	Probiotic group	Control group	Difference (%)	Probiotic group	Control group	Difference (%)
	Number of animals			Number of animals		
Inserted	15,400	15,400	0	15,000	15,000	0
Sent to slaughter	14,452	14,621	1.16	14,153	14,374	1.54
Accepted animals for consumption	14,147	14,361	1.49	14,002	14,218	1.52
Mortality	Dead animals (% mortality)		Significant difference	Dead animals (% mortality)		Significant difference
In production	948 (6.16)	779 (5.06)	p<0.0001	847 (5.65)	626 (4.17)	p<0.0001
Transportation	50 (0.32)	31 (0.20)	p<0.05	11 (0.07)	3 (0.02)	p<0.05
Condemned	255 (1.66)	229 (1.49)	NS	140 (0.93)	153 (1.02)	NS
Total	1,253 (8.14)	1,039 (6.75)	p<0.0001	998 (6.65)	782 (5.21)	p<0.0001
Total weight	Total biomass (kg)		Difference (%)	Total biomass (kg)		Difference (%)
Total at slaughter facilities	36,705	36,017	1.91	36,047	35,327	2.04
Accepted for consumption	36,169	35,556	1.72	35,847	35,095	2.14
Mean weight	Mean weight per animal (kg)		Difference (%)	Mean weight per animal (kg)		Difference (%)
Total at slaughter facilities	2.54	2.46	3.25	2.55	2.46	3.66
Accepted for consumption	2.56	2.48	3.26	2.56	2.47	3.72

2 NS, not statistically significant

Table 4. Percentage of animals in the different condemnation categories.

Experiment	House	Treatment	Skin discoloration	Irregular color/odor/consistency	Ascites	Stunted growth	Entrails remaining	Lack of bleeding off
1	1	Probiotics	14.5	54.9	14.5	4.71	5.88	5.49
1	2	Control	19.7	53.7	7.86	7.42	2.18	6.99
2	2	Probiotics	20.7	29.3	21.4	6.43	17.9	4.29
2	1	Control	14.4	35.9	15.7	1.31	26.1	6.54

Table 5. Production efficiency for experiments 1 and 2.

	Experiment 1		Experiment 2	
	Probiotic	Control	Probiotic	Control
Feed Conversion Ratio (FCR)	1.61	1.64	1.56	1.58
Production Efficiency Factor (PEF)	365	351	375	379
Adjusted FCR for animals accepted for consumption	1.70	1.73	1.66	1.64
Adjusted PEF for animals accepted for consumption	355	343	372	367