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Abstract: C. jejuni is the most worrisome food safety concern to both public health authorities and consumers since it is the leading bacterial cause of food-borne gastroenteritis in humans. A high incidence of C. jejuni in broiler flocks is often correlated to pathogen recovery in retail poultry meat, which is the main source of human infection. In this work broiler chickens were fed with a synbiotic product mixed with conventional feed using two different administration strategies. The synbiotic was formulated with the microencapsulated probiotic Bifidobacterium longum PCB133 and a xylo-oligosaccharide (XOS). 1-day old chicks were infected with C. jejuni M1 (106 cfu/ml) and the synbiotic mixture was then administered starting from the first and the 14th day of chicken life (for animal groups GrpC and GrpB respectively). The goal of this study was to monitor C. jejuni load at caecum level at different sampling time by Real Time PCR, identifying the best administration strategy. The microbiological analysis of the caecal content also considered the quantification of Campylobacter spp., Bifidobacterium spp. and B. longum.
The supplemented synbiotic was more successful in reducing C. jejuni and Campylobacter spp. when administered lifelong, compared to the shorter supplementation (GrpB). Bifidobacterium spp. quantification did not show significant differences among treatments and B. longum PCB133 was detected in both supplemented groups evidencing the successful colonization of the strain. Moreover, the samples of the control group (GrpA) and GrpC were analysed with PCR-denaturing gradient gel electrophoresis (PCR-DGGE) to compare the caecal microbial community profiles at the beginning and at the end of the trial. Pattern analysis evidenced the strong influence of the early synbiotic supplementation, although a physiological change in the microbial community, occurring during growth, could be observed. Experimental results demonstrate that the synbiotic approach at farm level can be an effective strategy, combined with biosecurity measures, to improve the safety of poultry meat.

Evidence of *Campylobacter jejuni* reduction in broilers with early synbiotic administration

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Abstract

C. jejuni is the most worrisome food safety concern to both public health authorities and consumers since it is the leading bacterial cause of food-borne gastroenteritis in humans. A high incidence of *C. jejuni* in broiler flocks is often correlated to pathogen recovery in retail poultry meat, which is the main source of human infection. In this work broiler chickens were fed with a synbiotic product mixed with conventional feed using two different administration strategies. The synbiotic was formulated with the microencapsulated probiotic *Bifidobacterium longum* PCB133 and a xylo-oligosaccharide (XOS). 1-day old chicks were infected with *C. jejuni* M1 (10^6 cfu/ml) and the synbiotic mixture was then administered starting from the first and the 14^{th} day of chicken life (for animal groups GrpC and GrpB respectively). The goal of this study was to monitor *C. jejuni* load at caecum level at different sampling time by Real Time PCR, identifying the best administration strategy. The microbiological analysis of the caecal content also considered the quantification of *Campylobacter* spp., *Bifidobacterium* spp. and *B. longum*.

The supplemented synbiotic was more successful in reducing *C. jejuni* and *Campylobacter* spp. when administered lifelong, compared to the shorter supplementation (GrpB). *Bifidobacterium* spp. quantification did not show significant differences among treatments and *B. longum* PCB133 was detected in both supplemented groups evidencing the successful colonization of the strain. Moreover, the samples of the control group (GrpA) and GrpC were analysed with PCR-denaturing gradient gel electrophoresis (PCR-DGGE) to compare the caecal microbial community profiles at the beginning and at the end of the trial. Pattern analysis evidenced the strong influence of the early synbiotic supplementation, although a physiological change in the microbial community, occurring during growth, could be observed. Experimental results demonstrate that the synbiotic approach at farm level can be an effective strategy, combined with biosecurity measures, to improve the safety of poultry meat.

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1. Introduction

Monitoring of *Campylobacter jejuni* in broilers from hatching to slaughter is of fundamental importance to preserve consumer health, since transmission to humans could lead to severe consequences. Human campylobacteriosis is the most frequent zoonosis in the European Union with 236,851 confirmed cases in the year 2014 and broiler meat is the most common food vehicle associated with this disease (EFSA, 2015). In addition to gastrointestinal disorders, 1% of cases may develop peripheral neuropathies, including Guillain-Barré syndrome, reactive arthritis and functional bowel diseases such as irritable bowel syndrome (Epps et al., 2013; Spiller and Lam, 2012). Despite biosecurity measures, broiler houses show a high presence of *C. jejuni* in the chicken gut; in 2014, *Campylobacter* was found in 30.7% of the 13,603 units tested within the EU member states with percentages ranging from 70% to 92% in Greece, Portugal and United Kingdom (EFSA, 2015). *C. jejuni* is considered a gut commensal in chickens; however, Humphrey et al. (2014) have recently shown that some chicken breeds used in intensive production systems have a strong inflammatory response to *C. jejuni* infection leading to disease. The high incidence and the need to prevent zoonosis require a common effort to remove and reduce the pathogen load at farm level in order to lower the risk of transmission along the poultry meat chain. In addition to good hygienic practices during slaughtering and decontamination treatments of carcasses or meat products, feeding probiotic/synbiotic supplements to broilers could contribute to food safety from the initial step of the "farm to fork" food chain (Gaggìa et al., 2010). Probiotic microorganisms (*e.g. Lactobacillus* and *Bifidobacterium* strains), both alone or combined with prebiotic ingredients in a synbiotic mixture, can beneficially affect the host, maintaining a healthy gut microbiota in animals and reducing the risk of pathogen infection (Allen et al., 2013; Gaggìa et al., 2010). The combination with prebiotics is considered an effective strategy, taking into account that such ingredients are selectively fermented in the colonic environment by probiotics, thus stimulating their growth (Gibson et al., 2004). This is an important feature in high intensive flocks where dietary changes during chicken lifespan and therapeutic antibiotics may strongly alter the composition of these

microbial groups, increasing the susceptibility to gastrointestinal infection and the shedding of food-borne pathogens (Bomba et al., 2002; Oliver et al., 2009). In the last decades, the use of synbiotic supplements in poultry flocks has been largely investigated; however, their efficacy is not fully established because of the variability of the experimental settings. Lactobacilli and bifidobacteria are the most used probiotic strains in animal feeding, combined with galacto-oligosaccharides (GOS), fructo-oligosaccharadies (FOS) or xilo-oligosaccharides (XOS). Studies usually reported the improving of growth parameters (weight, feed intake etc.), the modulation of the gut microbiota with the increase of beneficial microbial groups and the reduction of the load of pathogens such as *C. jejuni* and *Salmonella enterica* (Baffoni et al., 2012; Gaggìa et al., 2010; Santini et al., 2010).

This work aimed at evaluating the impact of a synbiotic formula in broilers challenged with *C. jejuni* strain M1, a virulent strain capable of direct transmission from poultry source to humans (Friis et al., 2010). The formula, composed by the microencapsulated *B. longum* subsp. *longum* PCB133 and a xylo-oligosaccharide (XOS), was administered to chicks from the first and the 14th day of chicken life, and its efficacy was evaluated by monitoring *Campylobacter* spp., *Bifidobacterium* spp. and *C. jejuni* in the caecal content by real-time PCR. The analysis of *Campylobacter* spp. was also supported by conventional microbiology. Moreover, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) was performed to evaluate the caecal microbial community.

2. Materials and Methods

2.1 Synbiotic composition

The synbiotic supplement consisted of the probiotic strain *B. longum* subsp. *longum* PCB133 (Santini et al., 2010) and the prebiotic xylooligosaccharide. The bacterial strain, microencapsulated in a lipid matrix according to Baffoni et al. (2012), was purchased by Probiotical S.p.A. (Milan, Italy) at a concentration of 10^9 cfu/g and was added to feed at 1% (w/w). The prebiotic

oligosaccharide was a 35% xylooligosaccharide (XOS35P) purchased from Italfeed s.r.l. (Milan, Italy) and added to feed at 0.2% (w/w).

2.2 Infection, animal management and sampling

C. jejuni strain M1 was used to infect animals and was provided by the University of Liverpool (Chaloner et al., 2014). One hundred twenty 1-day old chicks, obtained directly from hatchery and tested negative for *Campylobacter*, were divided into three groups. All groups were immediately infected by oral gavages with a 0.1 ml solution containing a challenge dose of *C. jejuni* M1 (10^6 cfu/ml).

The three groups of forty animals were named and managed as follows: 1) GrpA - chickens fed *ad libitum* with conventional feed; 2) GrpB - chickens fed *ad libitum* with conventional feed supplemented with the synbiotic product from the 14^{th} day of life; 3) GrpC - chickens fed *ad libitum* with conventional feed supplemented with the synbiotic product starting from the first day of life. The conventional feed is described in Table 1. Birds were reared under hygienic management practices throughout the entire period of the study. During the experiment, breeding conditions in terms of equipment, temperature and hours of daylight were conventionally set.

Four animals per each group were slaughtered at day 5 after infection to evaluate *C. jejuni* colonization. Nine/ten broilers belonging to each group were slaughtered at 10, 20, 30, 39 days of life (sampling times ST1, ST2, ST3 and ST4, respectively) and caecal content collected for the microbiological analysis and DNA extraction. Experiments were conducted according to animal welfare and protection (directive no. 86/609/EEC and Italian Law Act, Decreto Legislativo no. 116, issued on 27 January 1992).

2.2 Campylobacter spp. enumeration from caeca with plate count analysis

Campylobacter spp. detection and enumeration was carried out from 1 g of caecal content according to ISO standard 10272:2006 (Part 2). Ten fold serial dilutions were set up and each

dilution was plated on Karmali Agar (Oxoid, Milan, Italy) and incubated in microaerophilic conditions at 42 °C. Following incubation, the number of colony forming units per gram (cfu/g) of caecal content was recorded and means and standard deviations were calculated.

2.3 DNA extraction

Genomic DNA was extracted from caecal digesta by using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer instructions. The recommended lysis temperature was increased to 95 °C. Quantification of extracted DNA was performed spectrophotometrically (Infinite® 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland). The DNA was stored at −20 °C until use.

2.4 Real Time PCR

Quantification of target microorganisms (*Campylobacter* spp., *C. jejuni, Bifidobacterium* spp., *B. longum*) was carried out on DNA extracted from caecal content of ten animals belonging to GrpA, GrpB and GrpC at sampling times ST1, ST2, ST3 and ST4. Analysis was performed according to Baffoni et al. (2012) using a StepOne™ RealTime PCR system (Applied Biosystems, Foster City, CA, USA). Data were transformed to obtain the number of bacterial cells/g caecal content according to the rRNA gene copy number available at The Ribosomal RNA Database (rrDB, Klappenbach et al., 2001; Lee et al., 2009).

2.5 PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

Bacterial DNA was amplified by PCR with the primer pair HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA-3′) (Walter et al., 2000). DGGE analysis on obtained amplicons was performed as described by Muyzer et al. (1993), using a DCode System apparatus (Bio-Rad). Polyacrylamide gels [7% (w/v) acrylamide:bisacrylamide (37.5:1) (Bio-Rad)]

157 in 1× Tris–Acetate–EDTA (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System
158 (Model 475, Bio-Rad), with solutions containing 35–60% denaturant [100% denaturant corresponds
159 to 7 M urea (Sigma–Aldrich) and 40% (v/v) formamide (Sigma–Aldrich)]. The electrophoresis was
160 run at 55 V for 16 h at 60 °C. Gels were stained in a solution of 1× SYBR-Green (Sigma–Aldrich)
161 in 1× TAE for 20 min and their images captured in UV transillumination with Gel DocTM 226 XR
162 apparatus (Bio-Rad). Patterns were normalized by including a ladder with PCR products obtained
163 from known pure cultures. A cluster analysis was carried out by unweighted pair group method with
164 arithmetic mean (UPGMA) algorithm based on the Pearson correlation coefficient with an
165 optimization coefficient of 1% (Gel Compare software, version 6.6; Applied Maths, Sint-Martens-
166 Latem, Belgium). Microbial diversity was analyzed with Gel Compare 6.6 for the following
167 parameters: species richness, determined by the index (d) proposed by Menhinick (1964); Shannon-
168 Wiener index (H) and band evenness (EH), calculated according to Hill et al. (2003). Relevant
169 bands migrating at the same distance of the reference microorganisms in the ladder (*C. jejuni* M1
170 and *B. longum* PCB133) were cut from the gel and processed for sequencing (Eurofins genomics,
171 Ebersberg, Germany) according to Gaggìa et al. (2013). Sequence chromatograms were edited and
172 analyzed using the software programs Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA).
173 Final sequences were aligned with T-Coffee software (Notredame et al., 2000) and subjected to
174 taxon classification using reference sequences from Greengenes (DeSantis et al., 2006).
175
176 *2.6 Statistical analysis*
177 Statistical analysis was performed with R software using the following packages: lattice, graphics,
178 lawstat, car, agricolae, foreign, dunn.test, ggplot2 (R Core Team, 2016). Normal and homoscedastic
179 data were analyzed with two-way ANOVA and Tukey post-hoc test with Bonferroni correction.
180 Non-normal homoscedastic data were analyzed with non parametric Kruskall-Wallis test and
181 Dunn's post-hoc test with Bonferroni correction.
182

3. Results

3.1. Campylobacter enumeration from caeca with plate count analysis

Campylobacter spp. enumeration in the different groups and sampling times is listed in Table 2. No significant differences were evidenced among treatments with values ranging from 3.33±2.15 to 8.36±0.58 log cfu/g. However, all groups showed a decrease at ST4 which is significant in GrpB and GrpC ($p < 0.05$) compared to their respective starting value (ST1).

3.2. Real Time PCR quantification

Quantification results of *Campylobacter* spp., *C. jejuni, Bifidobacterium* spp. and *B. longum* by Real Time PCR are shown in Table 2 and in Fig. 1. Two way ANOVA evidenced a significant interaction of time/treatment on *Campylobacter* spp. count. Post-hoc analysis showed that GrpC mean was different ($p<0.01$) compared to the other groups and, in particular, its counts were significantly lower at ST1 and ST4 compared to GrpA ($p=0.014$ and $p<0.01$, respectively).

The same trend was observed in *C. jejuni* data (Table 2 and Fig. 1b) in which statistical analysis confirmed a significant difference among groups (Kruskal-Wallis test; $p<0.01$). Also in this case, the post hoc test evidenced the GrpC as the group with an average count significantly lower compared to GrpA and GrpB ($p<0.01$). GrpA and GrpB counts were stable over time with a slight but not significant decrease at ST4, while *C. jejuni* count at ST1 in GrpC showed a significant reduction compared to ST1 in GrpA ($p<0.05$).

Two way ANOVA of *Bifidobacterium* spp. quantification showed a significant difference ($p<0.01$) among groups and pair-wise comparisons evidenced that bifidobacteria counts were significantly higher in the control group and GrpC compared to GrpB ($p<0.01$). No significant intra-group variability among sampling times could be observed. The administered probiotic strain PCB133 was detected in both GrpB and GrpC with values in the range 4.73-6.62 log cfu/g, revealing a significant decrease in GrpC over time.

209 *3.3. DGGE analysis*

210 Based on Real Time PCR results, a PCR-DGGE analysis was carried out to compare the microbial
211 community profile of the caecal content of broilers belonging to group GrpA and GrpC at ST1 and
212 ST4, where quantification analysis highlighted the most interesting results. UPGMA dendrogram is
213 reported in Fig. 2. Profiles of the bacterial communities in the treated group (GrpC) and the DNA
214 sequences of excised bands are shown in Fig. 3.

215 A clear separation was observed between GrpA and GrpC (similarity less than 10%). ST1 and ST4
216 within the treated group GprC fell into two separate clusters (similarity<28.4%), whereas GrpA
217 profiles at ST1 and ST4 were not completely separated and shared 40.39% similarity. The species
218 richness was significantly ($p<0.05$) lower in GrpC compared to GrpA at ST1 (0.45±0.08 and
219 0.54±0.07, respectively), whereas no significant difference was found at ST4 (0.65±0.06 and
220 0.71±0.08, respectively). In the same way, the Shannon–Wiener diversity index and the evenness in
221 GrpC (0.96±0.09; 0.76±0.04) was significantly lower compared to GrpA at ST1 (1.16±0.09;
222 0.82±0.04) ($p < 0.05$), whereas no significant differences were observed at ST4 (Shannon-Wiener:
223 1.29±0.05 vs 1.34±0.06; evenness: 0.85±0.03; 0.82±0.02). The excised bands, at the same migration
224 distance of *C. jejuni* M1 in the ladder (Fig. 3), were sequenced and aligned revealing 100%
225 similarity among them. The longest sequence is reported in Fig. 3 and was identified as *E.*
226 *coli*/*Shigella* group with 100% similarity. The bands migrating at the same distance of *B. longum*
227 PCB133 were identified as *B. longum* (97.8% similarity, sequence is reported in Fig. 3).

228

229 **4. Discussion**

230 *C. jejuni* positive broiler flocks have been frequently correlated with the contamination of carcasses
231 surface during the slaughter process and pathogen recovery in retail poultry meat (Rosenquist et al.,
232 2003). Undoubtedly, this represents an important public health concern due to the risk of pathogen
233 transmission to humans. Therefore, a lower level of *C. jejuni* at farm level may improve poultry
234 meat quality, reducing the impact on consumer's health. The use of probiotics and prebiotics,

235 combined with general biosecurity strategies at farm level and hygienic measures at slaughtering,
236 may represent an important tool to control *C. jejuni* load (Gaggìa et al., 2010, Gaggìa et al., 2011).
237 In this work, the probiotic strain *B. longum* PCB133 was employed taking into consideration the
238 encouraging results achieved in previous studies (Baffoni et al.2012; Santini et al. 2010), which
239 showed a significant reduction of *C. jejuni* in faeces of naturally infected chickens following a 15-
240 day supplementation. The *in vivo* trial here described is therefore a follow-up of the previous ones,
241 involving a higher number of animals, a challenge with *C. jejuni* and two different administration
242 times (lifelong and starting from the 3rd week of life until slaughtering); in addition the
243 microbiological analyses were performed on caecal contents. The infection was carried out with
244 *Campylobacter jejuni* M1, which is a strain with a documented case of direct transmission from
245 chicken to humans, causing enteritis (Friis et al., 2010; Sheppard et al., 2010). As Baffoni et al.
246 (2012), the probiotic strain was delivered mixed to feed and microencapsulated in a lipid matrix,
247 rather than frozen, to improve its survival both in the feeder and in the poultry gastrointestinal tract
248 (GIT). This also would allow a more effective use by the poultry farmer industry. The prebiotic
249 GOS (Baffoni et al., 2012) was replaced with a XOS, because of the difficulties encountered in
250 GOS supply at a low cost. The commercial XOS was selected based on literature data that showed
251 the stimulating effect of this oligosaccharide on the growth of bifidobacteria (Mäkeläinen et al.,
252 2010; Wang et al., 2010). The prebiotic amount in the feed (0.2%) was chosen testing its
253 fermentability at 0.2% and 0.4% (w/v). Results evidenced that the probiotic growth was comparable
254 to glucose, without significant difference between the two tested concentrations (data not shown).
255 To the best of our knowledge, this is the first work evaluating the effect of different administration
256 timing of a synbiotic formula on *C. jejuni* reduction.
257 Real Time PCR results targeting *Campylobacter* spp. and *C. jejuni* firstly evidenced the importance
258 of an early synbiotic administration. Real Time and plate count results on *Campylobacter* spp.
259 resulted comparable in each group, particularly at ST1, ST2 and ST3, while at ST4 a significant
260 difference ($p < 0.01$) was observed between the two methods, with Real Time PCR data resulting

higher than those obtained by plate counting. The discrepancy between the culture-dependent and independent approach is also reported in other studies (Josefsen et al. 2010; Melero et al. 2011), and can be explained with the higher sensitivity of molecular methods, which also detect viable but not cultivable and injured cells not always detectable through conventional microbiology. Moreover, the universal primers for *Campylobacter* spp. target all *Campylobacter* species, whereas the medium Karmali Agar is a blood free selective medium for the isolation of *C. jejuni* and *C. coli* at 42 °C. From this perspective, the combination of classical and molecular investigations could provide a more reliable picture of *C. jejuni* prevalence. Real Time PCR also evidenced the detection of PCB133 in all treated animals, in agreement with bands identification in the DGGE profiles, with the highest concentration of the probiotic strain in GrpC at ST1 associated with the lowest *C. jejuni* detection. Therefore, the administered probiotic strain is able to persist within the poultry GIT and to exert antimicrobial activities. Moreover, the synbiotic product seems to influence the composition and complexity of the whole caecal microbial community in young chicks, as revealed by the cluster analysis and the diversity indices. Nevertheless, commercial broilers are normally subjected to clear successional changes in the caecal taxonomic composition during the life cycle, which is associated with time and diet change (Oakley et al., 2014). The population succession usually occurs from a simple and transient community to one of increasing diversity in growing and aged broilers (Ranjitkar et al., 2016) and the obtained patterns profiles of both GrpA and GrpC broilers confirmed this natural trend. As the community becomes more complex, the administered PCB133 strain is no more detectable as a band in the GrpC profiles. As reported by Marzorati et al. (2008) this could be associated with the lower DNA concentration of the probiotic strain, among the total 16S rRNA gene sequences, which is not sufficiently amplified to be visualized on DGGE gel. However, Real Time data confirmed its presence also at ST4 and its efficacy, in combination with the prebiotic XOS, to reduce *C. jejuni* population. Another interesting finding comes from the autochthonous bifidobacteria in GrpA, which are not able to contrast *C. jejuni* infection, although their average number is comparable to GrpC. In this case it was not possible to appreciate a

significant stimulation of bifidobacteria by the prebiotic fiber as in Baffoni et al. (2012) with GOS. However, its presence could have a role in pathogen counteraction as already reported by Eeckhaut et al. (2008).

In conclusion, *B. longum* PCB133 associated with XOS is more effective in reducing *C. jejuni* at the beginning of animal life when the gut microbiota is still under development and more susceptible to changes. The most disputable issue concerns the economic aspect since a lifelong supplementation is undoubtedly more expensive, and farmers could be discouraged from using these additives. This work has also highlighted the need for further research on strain delivery methods to ensure the best performance of these additives in intensive farming and, at the same time, to lower the cost of the products. The testing of this synbiotic formulation in intensive farming conditions is actually in process.

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442 **Figure Captions**

443 Fig.1. Box-plots on Real-Time PCR data for *Campylobacter* spp. (a), *C. jejuni* (b), *Bifidobacterium*
444 spp. (c) and *B. longum* (d).

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446 Fig. 2. Cluster analysis of DGGE profiles of GrpA and GrpC at ST1 and ST4.

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448 Fig. 3 DGGE gel of GrpC at the first and last sampling time (ST1 and ST4). Squares evidence the
449 excised bands and asterisks show the respective sequences of the analysed bands.

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469 Table 1. Diet composition of reared chickens

Days	Type of feed	Appearance	Composition
1-10	Starter feed	Chopped	Corn, genetically modified soy flour of extraction decorticated and toasted , wheat, seed toasted soybeans genetically modified, maize gluten genetically modified, animal fats, sunflower meal of extraction, peas, hydrolysed pork protein, dicalcium phosphate, calcium carbonate, sodium chloride, sodium bicarbonate, nutritional additives, vitamins, provitamins, trace elements, digestibility enhancers, coccidiostats
11-20	Grower feed (type 1)	Pelleted	Corn, genetically modified soy flour of extraction decorticated and toasted, wheat, seed toasted soybeans genetically modified, maize gluten genetically modified, decorticated sunflower flour extraction, animal fats, peas, hydrolysed pork protein, dicalcium phosphate, calcium carbonate, sodium chloride, sodium bicarbonate, nutritional additives, vitamins, provitamins, trace elements, nutritional additives, amino acids and their salts, digestibility enhancers, coccidiostats
21-30	Grower feed (type 2)	Pelleted	Wheat, corn, genetically modified soy flour of extraction decorticated and toasted, seed toasted soybeans genetically modified, wheat in grains, animal fats, decorticated sunflower flour extraction, peas, dicalcium phosphate, calcium carbonate, sodium chloride, sodium bicarbonate, nutritional additives, vitamins, provitamins, trace elements, nutritional additives, amino acids and their salts, digestibility enhancers, coccidiostats
31-39	Finisher feed	Pelleted	Wheat, seed toasted soybeans genetically modified, genetically modified soy flour of extraction decorticated and toasted, maize, wheat in grains, animal fats, decorticated sunflower flour extraction, peas, dicalcium phosphate, calcium carbonate, sodium chloride, sodium bicarbonate, nutritional additives, vitamins, provitamins, trace elements, nutritional additives, amino acids and their salts, digestibility enhancers

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Table 2. Real-time PCR and plate count results for the investigated species and genera. GrpA: control group; GrpB group supplemented with the synbiotic product starting from the 14th day-of-life; GrpC: group lifelong supplemented with the synbiotic product

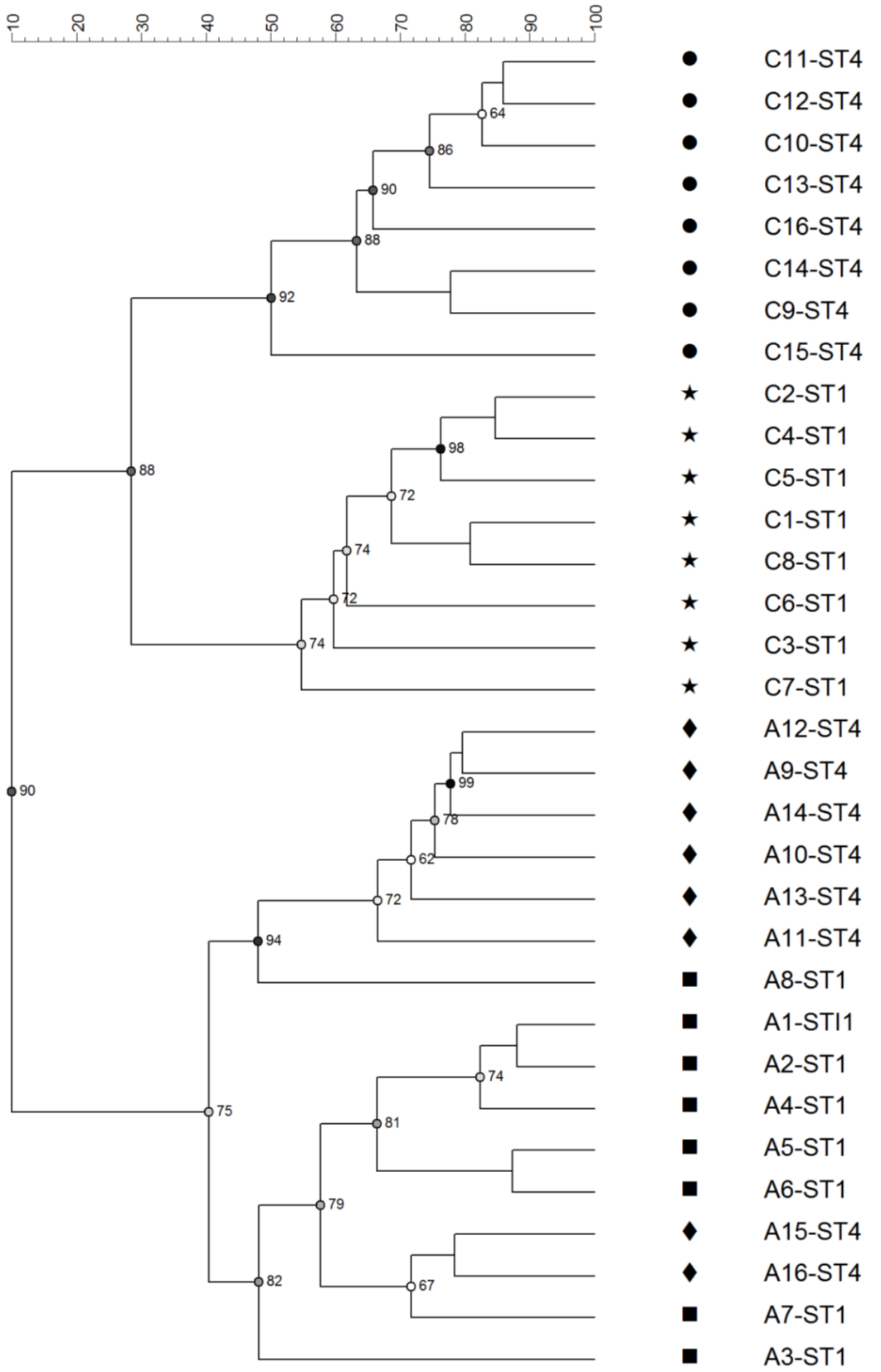
		***Campylobacter* spp.**		***C. jejuni***	***Bifidobacterium* spp.**	***B. longum***
		Real-time	Plate count	Real-time	Real-time	Real-time
GrpA	ST1	8.45±0.87 ^{ab}	7.64±0.5 ^{abc}	7.85±1.31 ^{ab}	6.67±0.85 ^{abc}	n.d.
	ST2	7.86±0.64 ^{abc}	8.36±0.58 ^{a}	7.45±0.92 ^{abcd}	6.66±0.57 ^{abcd}	n.d.
	ST3	8.22±0.91 ^{abc}	7.19±2.24 ^{abc}	8.03±1.41 ^{ab}	6.49±0.53 ^{abcde}	n.d.
	ST4	8.1±0.54 ^{abc}	5.67±2.00 ^{bcde}	7.59±0.92 ^{abc}	6.95±0.41 ^{a}	n.d.
GrpB	ST1	7.69±1.06 ^{abc}	8.03±0.48 ^{ab}	7.11±1.78 ^{abc}	5.89±0.93 ^{cde}	n.d.
	ST2	7.77±0.74 ^{abc}	8.05±0.92 ^{ae}	7.26±1.01 ^{abc}	5.86±0.35 ^{de}	4.73±0.81 ^{b}
	ST3	8.65±1.23 ^{a}	7.31±0.28 ^{ad}	8.34±1.37 ^{ad}	6.09±0.33 ^{cde}	5.35±0.57 ^{ab}
	ST4	7.52±0.92 ^{abc}	4.18±2.69 ^{cd}	6.22±1.92 ^{bc}	5.76±0.17 ^{e}	4.81±0.40 ^{b}
GrpC	ST1	6.94±0.34 ^{cd}	8.13±0.48 ^{a}	5.45±0.90 ^{c}	6.54±0.19 ^{abcd}	6.62±0.33 ^{a}
	ST2	7.72±0.37 ^{abc}	7.91±0.47 ^{ab}	7.60±0.38 ^{bcd}	6.60±0.46 ^{abcd}	6.33±0.91 ^{a}
	ST3	7.40±1.04 ^{bc}	6.62±2.21 ^{abcd}	6.55±1.92 ^{bcd}	6.16±0.18 ^{bcde}	5.43±1.02 ^{ab}
	ST4	6.20±0.61 ^{d}	3.33±2.15 ^{d}	5.79±1.04 ^{c}	6.71±0.45 ^{ab}	5.09±0.96 ^{b}

different letters mean statistical significance $p<0.05$
n.d. not detected

Figure 1

Campylobacter spp. log10 (cfu/g)
GrpA.ST1
GrpB.ST1
GrpC.ST1
GrpA.ST2
GrpB.ST2
GrpC.ST2
GrpA.ST3
GrpB.ST3
GrpC.ST3
GrpA.ST4
GrpB.ST4
GrpC.ST4
a)
C. jejuni log10 (cfu/g)
b)
Bifidobacterium spp. log10 (cfu/g)
c)
B. longum log10 (cfu/g)
d)

Figure 2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82
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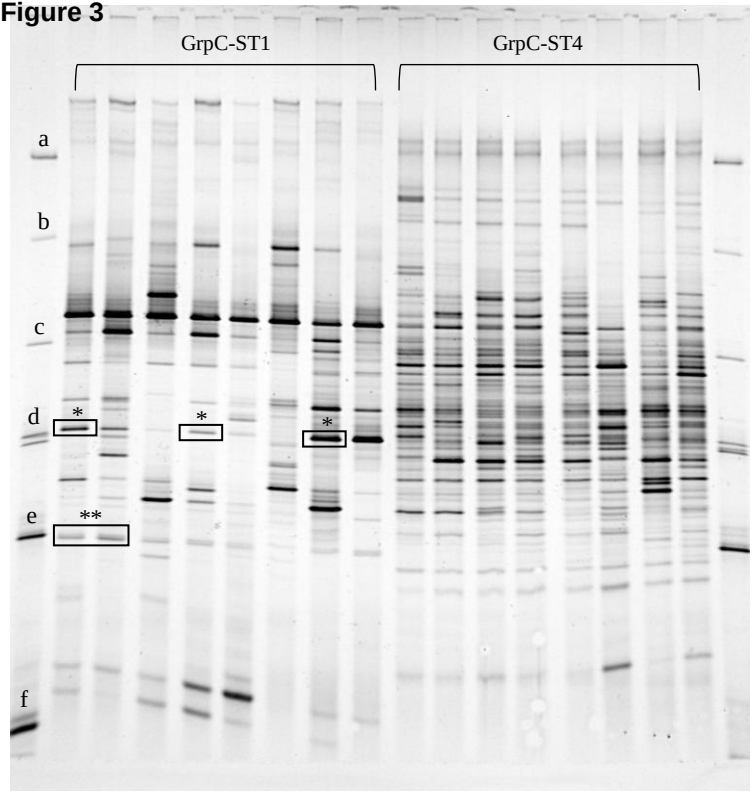
C11-ST4
C12-ST4
C10-ST4
C13-ST4
C16-ST4
C14-ST4
C9-ST4
C15-ST4
C2-ST1
C4-ST1
C5-ST1
C1-ST1
C8-ST1
C6-ST1
C3-ST1
C7-ST1
A12-ST4
A9-ST4
A14-ST4
A10-ST4
A13-ST4
A11-ST4
A8-ST1
A1-STI1
A2-ST1
A4-ST1
A5-ST1
A6-ST1
A15-ST4
A16-ST4
A7-ST1
A3-ST1

Figure 3



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TAAAGTTAATACCTTTGCTCATTGA
CGTTACCCGCAG3’

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5’TACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCGACGCCGCG
TGAGGGATGGAGGCCTTCGGGTTG
TAAACCTCTTTTATCGCGGGCAGC
AAGCGAGAGTGAGTTTACCCGT3’