Evidence of Campylobacter jejuni reduction in broilers with early synbiotic administration

BAFFONI, Loredana; GAGGIA, Francesca; GAROFOLO, Giuliano; DI SERAFINO, Gabriella; BUGLIONE, Enrico; DI GIANNATALE, Elisabetta; DI GIOIA, Diana

Evidence of Campylobacter jejuni reduction in broilers with early synbiotic administration

which has been published in final form in *INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY* Volume 251, 19 June 2017, Pages 41-47

The final published version is available online at:
https://doi.org/10.1016/j.ijfoodmicro.2017.04.001

Elsevier Editorial System(tm) for
International Journal of Food Microbiology
Manuscript Draft

Manuscript Number:

Title: Evidence of Campylobacter jejuni reduction in broilers with early synbiotic administration

Article Type: Full Length Article

Keywords: synbiotic; infection; Campylobacter jejuni; broiler; chicken microbiota; food safety

Corresponding Author: Dr. Diana Di Gioia, Ph.D.

Corresponding Author's Institution: University of Bologna

First Author: Loredana Baffoni

Order of Authors: Loredana Baffoni; Francesca Gaggìa; Giuliano Garofolo; Gabriella Di Serafino; Enrico Buglione; Elisabetta Di Giannatale; Diana Di Gioia, Ph.D.

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

Loredana Baffoni[a], Francesca Gaggìa[a], Giuliano Garofolo[b], Gabriella Di Serafino[b], Enrico Buglione[a1], Elisabetta Di Giannatale[b], Diana Di Gioia[a*]

[a]Department of Agricultural Sciences, University of Bologna, viale Fanin 44, 40127 Bologna, Italy

[b]Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', National Reference Laboratory for *Campylobacter*, Via Campo Boario, 64100 Teramo, Italy

[1]Present address: Laboratory of Neuroscience "R. Levi-Montalcini", Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milano, Italy

[*]corresponding author. Tel.: +390512096269; Fax: +390512096274. E-mail address: diana.digioia@unibo.it (D. Di Gioia)

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 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.

Keywords: synbiotic; infection; *Campylobacter jejuni*; broiler; chicken microbiota; food safety

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 14th 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)]

in 1× Tris–Acetate–EDTA (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad), with solutions containing 35–60% denaturant [100% denaturant corresponds to 7 M urea (Sigma–Aldrich) and 40% (v/v) formamide (Sigma–Aldrich)]. The electrophoresis was run at 55 V for 16 h at 60 °C. Gels were stained in a solution of 1× SYBR-Green (Sigma–Aldrich) in 1× TAE for 20 min and their images captured in UV transillumination with Gel DocTM 226 XR apparatus (Bio-Rad). Patterns were normalized by including a ladder with PCR products obtained from known pure cultures. A cluster analysis was carried out by unweighted pair group method with arithmetic mean (UPGMA) algorithm based on the Pearson correlation coefficient with an optimization coefficient of 1% (Gel Compare software, version 6.6; Applied Maths, Sint-Martens-Latem, Belgium). Microbial diversity was analyzed with Gel Compare 6.6 for the following parameters: species richness, determined by the index (d) proposed by Menhinick (1964); Shannon-Wiener index (H) and band evenness (EH), calculated according to Hill et al. (2003). Relevant bands migrating at the same distance of the reference microorganisms in the ladder (*C. jejuni* M1 and *B. longum* PCB133) were cut from the gel and processed for sequencing (Eurofins genomics, Ebersberg, Germany) according to Gaggìa et al. (2013). Sequence chromatograms were edited and analyzed using the software programs Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA). Final sequences were aligned with T-Coffee software (Notredame et al., 2000) and subjected to taxon classification using reference sequences from Greengenes (DeSantis et al., 2006).

2.6 Statistical analysis

Statistical analysis was performed with R software using the following packages: lattice, graphics, lawstat, car, agricolae, foreign, dunn.test, ggplot2 (R Core Team, 2016). Normal and homoscedastic data were analyzed with two-way ANOVA and Tukey post-hoc test with Bonferroni correction. Non-normal homoscedastic data were analyzed with non parametric Kruskall-Wallis test and Dunn’s post-hoc test with Bonferroni correction.

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.

3.3. DGGE analysis

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

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

4. Discussion

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

combined with general biosecurity strategies at farm level and hygienic measures at slaughtering, may represent an important tool to control *C. jejuni* load (Gaggìa et al., 2010, Gaggìa et al., 2011). In this work, the probiotic strain *B. longum* PCB133 was employed taking into consideration the encouraging results achieved in previous studies (Baffoni et al.2012; Santini et al. 2010), which showed a significant reduction of *C. jejuni* in faeces of naturally infected chickens following a 15-day supplementation. The *in vivo* trial here described is therefore a follow-up of the previous ones, involving a higher number of animals, a challenge with *C. jejuni* and two different administration times (lifelong and starting from the 3rd week of life until slaughtering); in addition the microbiological analyses were performed on caecal contents. The infection was carried out with *Campylobacter jejuni* M1, which is a strain with a documented case of direct transmission from chicken to humans, causing enteritis (Friis et al., 2010; Sheppard et al., 2010). As Baffoni et al. (2012), the probiotic strain was delivered mixed to feed and microencapsulated in a lipid matrix, rather than frozen, to improve its survival both in the feeder and in the poultry gastrointestinal tract (GIT). This also would allow a more effective use by the poultry farmer industry. The prebiotic GOS (Baffoni et al., 2012) was replaced with a XOS, because of the difficulties encountered in GOS supply at a low cost. The commercial XOS was selected based on literature data that showed the stimulating effect of this oligosaccharide on the growth of bifidobacteria (Mäkeläinen et al., 2010; Wang et al., 2010). The prebiotic amount in the feed (0.2%) was chosen testing its fermentability at 0.2% and 0.4% (w/v). Results evidenced that the probiotic growth was comparable to glucose, without significant difference between the two tested concentrations (data not shown). To the best of our knowledge, this is the first work evaluating the effect of different administration timing of a synbiotic formula on *C. jejuni* reduction.

Real Time PCR results targeting *Campylobacter* spp. and *C. jejuni* firstly evidenced the importance of an early synbiotic administration. Real Time and plate count results on *Campylobacter* spp. resulted comparable in each group, particularly at ST1, ST2 and ST3, while at ST4 a significant 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.

Acknowledgments

This work was supported by the Coordination of European Research on Emerging and Major Infectious Diseases of Livestock (EMIDA ERA-NET) (Dnro 776/312/2012) through the project entitled Biology and Control of *Campylobacter* in the Chicken Supply Chain (CamChain). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Allen, H.K., Levine, U.Y, Looft, T., Bandrick, M., Casey, T.A., 2013. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. Trends Microbiol. 21:114-119.

Baffoni, L., Gaggìa, F., Di Gioia, D., Santini, C., Mogna, L., Biavati, B., 2012. A *Bifidobacterium*-based synbiotic product to reduce the transmission of *C. jejuni* along the poultry food chain. Int. J. Food Microbiol. 157, 156-161.

Bomba, A., Nemcova, R., Mudronova, D., Guba, P., 2002. The possibilities of potentiating the efficacy of probiotics. Trends Food Sci. Tech. 13, 121-126.

Chaloner, G., Wigley, P., Humphrey, S., Kemmett, K., Lacharme-Lora, L., Humphrey, T., Williams, N., 2014. Dynamics of dual infection with *Campylobacter jejuni* strains in chickens reveals distinct strain-to-strain variation in infection ecology. Appl. Environ. Microbiol. 80, 6366-6372.

DeSantis, T.Z., Hugenholtz P., Larsen N., Rojas M., Brodie E.L., Keller K., Huber T., Dalevi D., Hu, P., Andersen G.L., 2006. Greengenes, a chimera-checked 16S rRNA Gene Database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069-5072.

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Courtin, C.M., Delcour, J.A., Broekaert, W.F., 2003. Arabinoxylooligosaccharides from wheat bran inhibit *Salmonella* colonization in broiler chickens. Poultry Sci. 8, 2329-2334.

EFSA, 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal 13, 4329.

Epps, S.V., Harvey, R.B., Hume, M.E., Phillips, T.D., Anderson, R.C., Nisbet, D.J., 2013. Foodborne *Campylobacter*: infections; metabolism; pathogenesis and reservoirs. Int. J. Environ. Res. Public Health. 10, 6292-6304.

Friis, C., Wassenaar, T.M., Javed, M.A., Snipen, L., Lagesen, K., Hallin, P.F., Newell, D.G., Toszeghy, M., Ridley, A., Manning, G., Ussery, D.W., 2010. Genomic characterization of *Campylobacter jejuni* strain M1. PLoS One 5:e12253

Gaggia, F., Mattarelli, P., Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe food products. Int. J. Food Microbiol. 141, 15-28.

Gaggìa F., Di Gioia D., Baffoni L., Biavati B., 2011. The role of protective and probiotic cultures in food and feed and their impact in food safety. Trends Food Sci. Tech. 22, S58-S66.

Gaggìa F., Baffoni L., Di Gioia D., Accorsi M., Bosi S., Marotti I., Biavati B., Dinelli G., 2013. Inoculation with effective microorganisms of *Lolium perenne* L.: evaluation of plant growth parameters and endophytic colonization of roots. New Biotechnol. 30, 695-704.

Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A., Roberfroid, M.B., 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17, 259-275.

Hill, T., Walsh, K., Harris, J., Moffett, B., 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiol. Ecol. 43, 1-11.

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., Humphrey,

T., Wigley, P., 2014. *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. MBio 5, e01364-14.

ISO. Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for Detection and Enumeration of *Campylobacter* spp. - Part 2: Enumeration Method (ISO 10272:2006).

Josefsen, M.H., Löfström, C., Hansen, T.B., Christensen, L.S., Olsen, J.E., Hoorfar, J., 2010. Rapid quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. Appl. Environ. Microbiol. 76:5097-5104.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrnDB: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29, 181-184.

Lee, Z.M., Bussema, C. 3rd, Schmidt, T.M., 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res. 37, 489-493.

Mäkeläinen, H., Saarinen, M., Stowell, J., Rautonen, N., Ouwehand, A.C., 2010. Xylo-oligosaccharides enhance the growth of bifidobacteria and *Bifidobacterium lactis* in a simulated colon model. Beneficial Microbes. 1, 81-91.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol. 10, 1571-1581.

Melero, B., Cocolin, L., Rantsiou, K., Jaime, I., Rovira, J., 2011. Comparison between conventional and qPCR methods for enumerating *Campylobacter jejuni* in a poultry processing plant. Food Microbiol. 28:1353-1358.

Menhinick, E.F., 1964. A comparison of some species-individuals diversity indices applied to samples of field insects. Ecology 45, 859-861.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.

Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302, 205-217.

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J., Cox, N.A., 2014. The chicken gastrointestinal microbiome. FEMS Microbiol. Lett. 360:100-112.

Oliver, S.P., Patel, D.A., Callaway, T.R., Torrence, M.E., 2009. ASAS Centennial Paper: developments and future outlook for preharvest food safety. J. Anim. Sci. 87, 419-437.

Ranjitkar, S., Lawley, B., Tannock, G., Engberg, R.M., 2016. Bacterial succession in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 82, 2399-2410.

R Core Team, 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rosenquist, H., Nielsen, N.L., Sommer, H.M., Nørrung, B., Christensen, B.B., 2003. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. Int. J. Food Microbiol. 83, 87-103.

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioa D., Biavati, B., 2010. Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. Int. J. Food Microbiol. 141, 98-108.

Sheppard, S.K, Colles, F., Richardson, J., Cody, A.J, Elson, R., Lawson, A., Brick, G., Meldrum, R., Little, C.L., Owen, R.J., Maiden, M.C.J., McCarthy, N.D., 2010. Host association of *Campylobacter* genotypes transcends geographic variation. Appl. Environ. Microbiol. 76, 5269-5277.

Spiller, R., Lam, C., 2012. An update on post-infectious irritable bowel syndrome: role of genetics; immune activation serotonin and altered microbiome. J. Neurogastroenterol. Motil. 18, 258-268.

Walter, J., Tannock, G.W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D.M., Munro, K., Alatossava, T., 2000. Detection and identification of gastro-intestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. 66, 297-303.

Wang, J., Baoguo Suna, B., Caoa, Y., Wanga, C., 2010. *In vitro* fermentation of xylooligosaccharides from wheat bran insoluble dietary fiber by bifidobacteria. Carbohyd. Polym. 82, 419-423.

Figure Captions

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

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

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

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

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

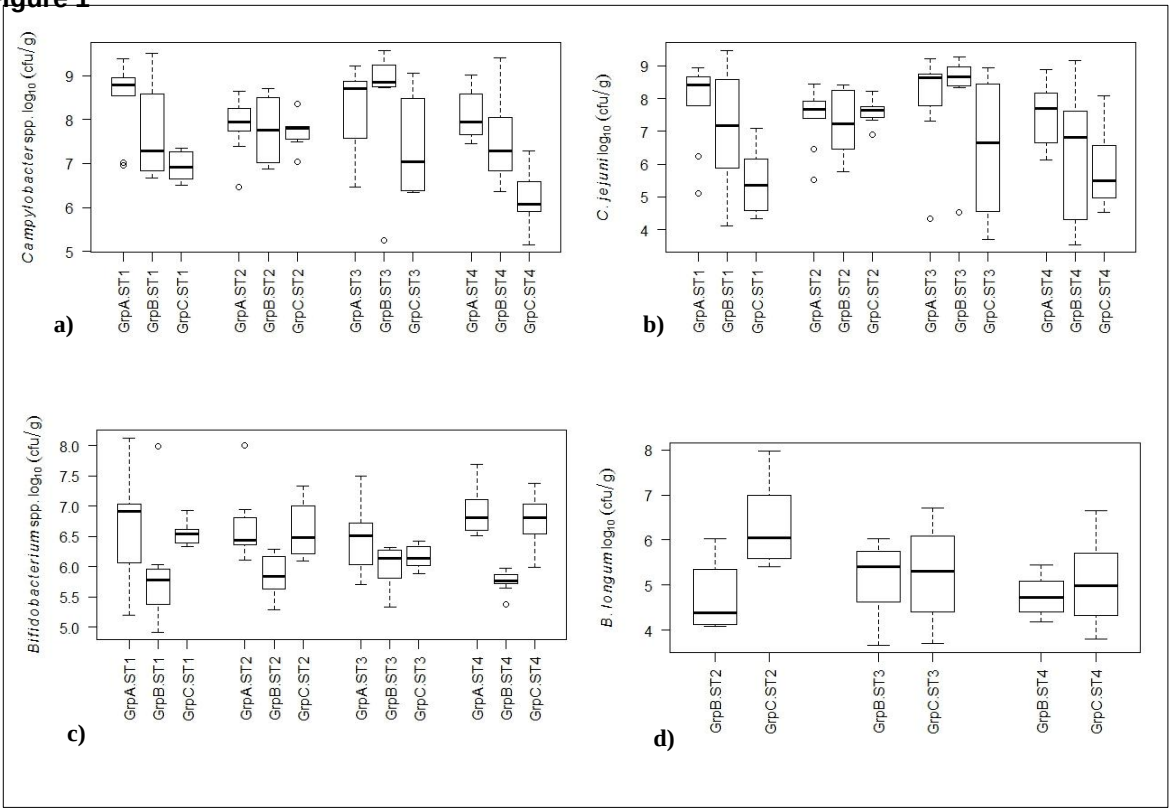

a)
Campylobacter spp. log10 (cfu/g)
GrpA.ST1
GrpB.ST1
GrpC.ST1
GrpA.ST2
GrpB.ST2
GrpC.ST2
GrpA.ST3
GrpB.ST3
GrpC.ST3
GrpA.ST4
GrpB.ST4
GrpC.ST4
b)
C. jejuni log10 (cfu/g)
c)
Bifidobacterium spp. log10 (cfu/g)
d)
B. longum log10 (cfu/g)

Figure 2

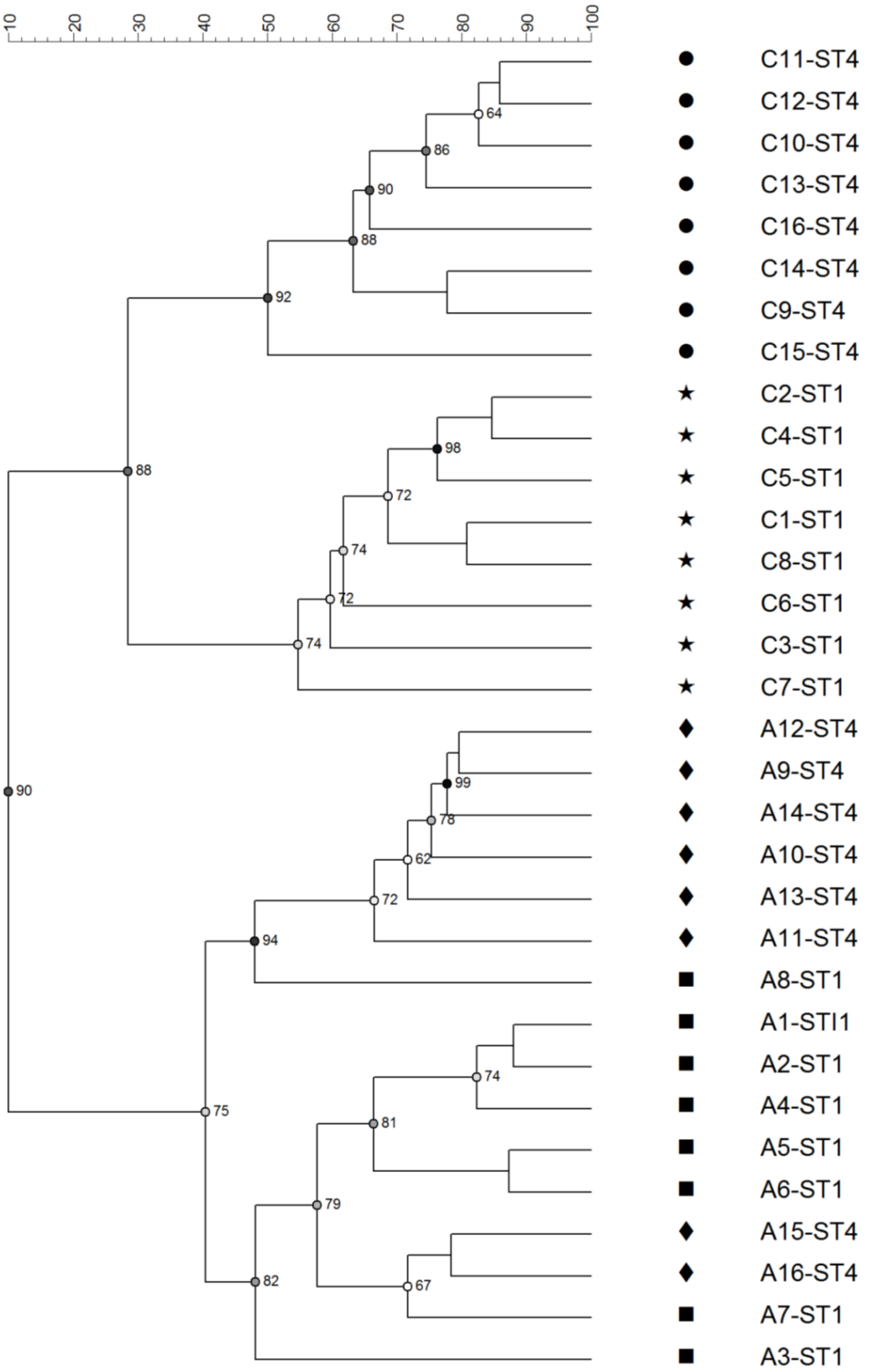
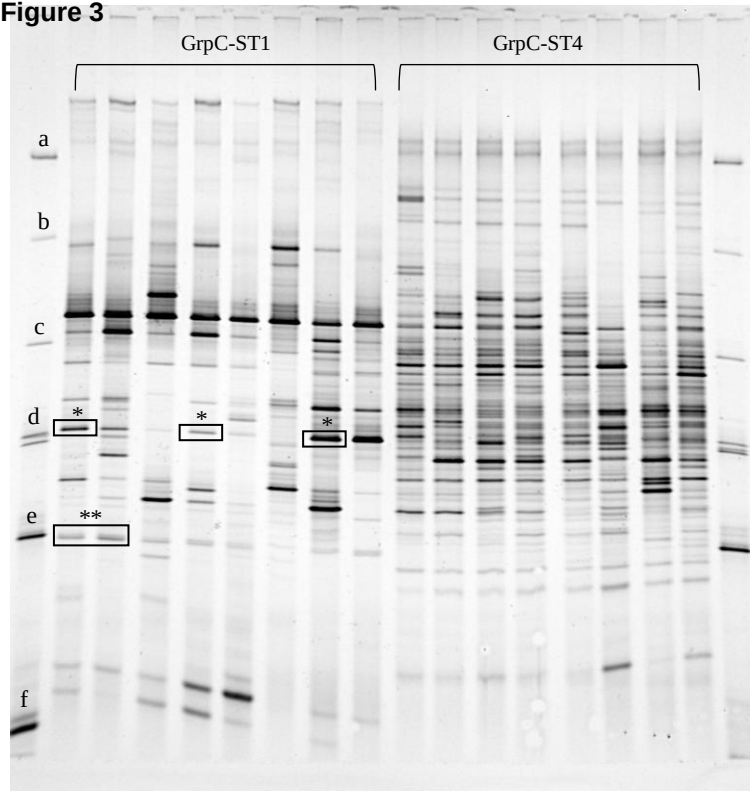

10
20
30
40
50
60
70
80
90
100
64
86
90
88
92
98
88
72
74
72
74
90
99
78
62
72
94
74
81
75
79
67
82
C11-ST4
C12-ST4
C10-ST4
C13-ST4
C16-ST4
C14-ST4
C9-ST4
C15-ST4
C2-ST1
C4-ST1
C5-ST1
C1-ST1
C8-ST1
C6-ST1
C3-ST1
C7-ST1
A12-ST4
A9-ST4
A14-ST4
A10-ST4
A13-ST4
A11-ST4
A8-ST1
A1-STI1
A2-ST1
A4-ST1
A5-ST1
A6-ST1
A15-ST4
A16-ST4
A7-ST1
A3-ST1

Figure 3



*

5’ACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAG
CCTGATGCAGCCATGCCGCGTGTAT
GAAGAAGGCCTTCGGGTTGTAAAG
TACTTTCAGCGGGGAGGAAGGGAG
TAAAGTTAATACCTTTGCTCATTGA
CGTTACCCGCAG3’

**

5’TACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCGACGCCGCG
TGAGGGATGGAGGCCTTCGGGTTG
TAAACCTCTTTTATCGCGGGCAGC
AAGCGAGAGTGAGTTTACCCGT3’