



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

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. - In: INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY. - ISSN 0168-1605. - ELETTRONICO. - 251:19 June 2017(2017), pp. 41-47. [10.1016/j.ijfoodmicro.2017.04.001]

Availability:

This version is available at: <https://hdl.handle.net/11585/584725> since: 2017-04-30

Published:

DOI: <http://doi.org/10.1016/j.ijfoodmicro.2017.04.001>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

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>

© 2017 Elsevier. This manuscript version is made available under the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) 4.0 International License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

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 Gaggia; 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.

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

2

3 **Loredana Baffoni^a, Francesca Gaggà^a, Giuliano Garofolo^b, Gabriella Di Serafino^b, Enrico**
4 **Buglione^{a1}, Elisabetta Di Giannatale^b, Diana Di Gioia^{a*}**

5

6 **^aDepartment of Agricultural Sciences, University of Bologna, viale Fanin 44, 40127 Bologna,**
7 **Italy**

8 **^bIstituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', National**
9 **Reference Laboratory for *Campylobacter*, Via Campo Boario, 64100 Teramo, Italy**

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

12

13

14

15

16

17

18

19

20

21

22

23

24

25 ***corresponding author. Tel.: +390512096269; Fax: +390512096274. E-mail address:**

26 **diana.digioia@unibo.it (D. Di Gioia)**

27 **Abstract**

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

40 The supplemented synbiotic was more successful in reducing *C. jejuni* and *Campylobacter* spp.
41 when administered lifelong, compared to the shorter supplementation (GrpB). *Bifidobacterium* spp.
42 quantification did not show significant differences among treatments and *B. longum* PCB133 was
43 detected in both supplemented groups evidencing the successful colonization of the strain.

44 Moreover, the samples of the control group (GrpA) and GrpC were analysed with PCR-denaturing
45 gradient gel electrophoresis (PCR-DGGE) to compare the caecal microbial community profiles at
46 the beginning and at the end of the trial. Pattern analysis evidenced the strong influence of the early
47 synbiotic supplementation, although a physiological change in the microbial community, occurring
48 during growth, could be observed. Experimental results demonstrate that the synbiotic approach at
49 farm level can be an effective strategy, combined with biosecurity measures, to improve the safety
50 of poultry meat.

51

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

53 **1. Introduction**

54 Monitoring of *Campylobacter jejuni* in broilers from hatching to slaughter is of fundamental
55 importance to preserve consumer health, since transmission to humans could lead to severe
56 consequences. Human campylobacteriosis is the most frequent zoonosis in the European Union
57 with 236,851 confirmed cases in the year 2014 and broiler meat is the most common food vehicle
58 associated with this disease (EFSA, 2015). In addition to gastrointestinal disorders, 1% of cases
59 may develop peripheral neuropathies, including Guillain-Barré syndrome, reactive arthritis and
60 functional bowel diseases such as irritable bowel syndrome (Epps et al., 2013; Spiller and Lam,
61 2012). Despite biosecurity measures, broiler houses show a high presence of *C. jejuni* in the chicken
62 gut; in 2014, *Campylobacter* was found in 30.7% of the 13,603 units tested within the EU member
63 states with percentages ranging from 70% to 92% in Greece, Portugal and United Kingdom (EFSA,
64 2015). *C. jejuni* is considered a gut commensal in chickens; however, Humphrey et al. (2014) have
65 recently shown that some chicken breeds used in intensive production systems have a strong
66 inflammatory response to *C. jejuni* infection leading to disease. The high incidence and the need to
67 prevent zoonosis require a common effort to remove and reduce the pathogen load at farm level in
68 order to lower the risk of transmission along the poultry meat chain. In addition to good hygienic
69 practices during slaughtering and decontamination treatments of carcasses or meat products, feeding
70 probiotic/synbiotic supplements to broilers could contribute to food safety from the initial step of
71 the “farm to fork” food chain (Gaggia et al., 2010). Probiotic microorganisms (*e.g. Lactobacillus*
72 and *Bifidobacterium* strains), both alone or combined with prebiotic ingredients in a synbiotic
73 mixture, can beneficially affect the host, maintaining a healthy gut microbiota in animals and
74 reducing the risk of pathogen infection (Allen et al., 2013; Gaggia et al., 2010). The combination
75 with prebiotics is considered an effective strategy, taking into account that such ingredients are
76 selectively fermented in the colonic environment by probiotics, thus stimulating their growth
77 (Gibson et al., 2004). This is an important feature in high intensive flocks where dietary changes
78 during chicken lifespan and therapeutic antibiotics may strongly alter the composition of these

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

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

98

99 **2. Materials and Methods**

100 *2.1 Synbiotic composition*

101 The synbiotic supplement consisted of the probiotic strain *B. longum* subsp. *longum* PCB133
102 (Santini et al., 2010) and the prebiotic xylooligosaccharide. The bacterial strain, microencapsulated
103 in a lipid matrix according to Baffoni et al. (2012), was purchased by Probiotical S.p.A. (Milan,
104 Italy) at a concentration of 10⁹ cfu/g and was added to feed at 1% (w/w). The prebiotic

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

107

108 *2.2 Infection, animal management and sampling*

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

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

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

127

128 *2.2 Campylobacter spp. enumeration from caeca with plate count analysis*

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

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

134

135 *2.3 DNA extraction*

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

141

142 *2.4 Real Time PCR*

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

150

151 *2.5 PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)*

152 Bacterial DNA was amplified by PCR with the primer pair HDA1-GC (5'-CGC CCG GGG CGC
153 GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-
154 3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA-3') (Walter et al., 2000). DGGE analysis
155 on obtained amplicons was performed as described by Muyzer et al. (1993), using a DCode System
156 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 Doc™ 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 Gaggia 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 Kruskal-Wallis test and
181 Dunn's post-hoc test with Bonferroni correction.

182

183 3. Results

184 3.1. *Campylobacter* enumeration from caeca with plate count analysis

185 *Campylobacter* spp. enumeration in the different groups and sampling times is listed in Table 2. No
186 significant differences were evidenced among treatments with values ranging from 3.33 ± 2.15 to
187 8.36 ± 0.58 log cfu/g. However, all groups showed a decrease at ST4 which is significant in GrpB
188 and GrpC ($p < 0.05$) compared to their respective starting value (ST1).

189

190 3.2. Real Time PCR quantification

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

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

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

208

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 GrpC 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 (Gaggia et al., 2010, Gaggia 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

261 higher than those obtained by plate counting. The discrepancy between the culture-dependent and
262 independent approach is also reported in other studies (Josefsen et al. 2010; Melero et al. 2011), and
263 can be explained with the higher sensitivity of molecular methods, which also detect viable but not
264 cultivable and injured cells not always detectable through conventional microbiology. Moreover,
265 the universal primers for *Campylobacter* spp. target all *Campylobacter* species, whereas the
266 medium Karmali Agar is a blood free selective medium for the isolation of *C. jejuni* and *C. coli* at
267 42 °C. From this perspective, the combination of classical and molecular investigations could
268 provide a more reliable picture of *C. jejuni* prevalence. Real Time PCR also evidenced the detection
269 of PCB133 in all treated animals, in agreement with bands identification in the DGGE profiles, with
270 the highest concentration of the probiotic strain in GrpC at ST1 associated with the lowest *C. jejuni*
271 detection. Therefore, the administered probiotic strain is able to persist within the poultry GIT and
272 to exert antimicrobial activities. Moreover, the synbiotic product seems to influence the
273 composition and complexity of the whole caecal microbial community in young chicks, as revealed
274 by the cluster analysis and the diversity indices. Nevertheless, commercial broilers are normally
275 subjected to clear successional changes in the caecal taxonomic composition during the life cycle,
276 which is associated with time and diet change (Oakley et al., 2014). The population succession
277 usually occurs from a simple and transient community to one of increasing diversity in growing and
278 aged broilers (Ranjitkar et al., 2016) and the obtained patterns profiles of both GrpA and GrpC
279 broilers confirmed this natural trend. As the community becomes more complex, the administered
280 PCB133 strain is no more detectable as a band in the GrpC profiles. As reported by Marzorati et al.
281 (2008) this could be associated with the lower DNA concentration of the probiotic strain, among the
282 total 16S rRNA gene sequences, which is not sufficiently amplified to be visualized on DGGE gel.
283 However, Real Time data confirmed its presence also at ST4 and its efficacy, in combination with
284 the prebiotic XOS, to reduce *C. jejuni* population. Another interesting finding comes from the
285 autochthonous bifidobacteria in GrpA, which are not able to contrast *C. jejuni* infection, although
286 their average number is comparable to GrpC. In this case it was not possible to appreciate a

287 significant stimulation of bifidobacteria by the prebiotic fiber as in Baffoni et al. (2012) with GOS.
288 However, its presence could have a role in pathogen counteraction as already reported by Eeckhaut
289 et al. (2008).
290 In conclusion, *B. longum* PCB133 associated with XOS is more effective in reducing *C. jejuni* at
291 the beginning of animal life when the gut microbiota is still under development and more
292 susceptible to changes. The most disputable issue concerns the economic aspect since a lifelong
293 supplementation is undoubtedly more expensive, and farmers could be discouraged from using
294 these additives. This work has also highlighted the need for further research on strain delivery
295 methods to ensure the best performance of these additives in intensive farming and, at the same
296 time, to lower the cost of the products. The testing of this synbiotic formulation in intensive farming
297 conditions is actually in process.

298

299 **Acknowledgments**

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

305

306

307

308

309

310

311

312

313 **References**

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

316

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

320

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

323

324 Chaloner, G., Wigley, P., Humphrey, S., Kemmett, K., Lacharme-Lora, L., Humphrey, T.,
325 Williams, N., 2014. Dynamics of dual infection with *Campylobacter jejuni* strains in chickens
326 reveals distinct strain-to-strain variation in infection ecology. Appl. Environ. Microbiol. 80, 6366-
327 6372.

328

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

332

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

336

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

339

340 Epps, S.V., Harvey, R.B., Hume, M.E., Phillips, T.D., Anderson, R.C., Nisbet, D.J., 2013.

341 Foodborne *Campylobacter*: infections; metabolism; pathogenesis and reservoirs. Int. J. Environ.

342 Res. Public Health. 10, 6292-6304.

343

344 Friis, C., Wassenaar, T.M., Javed, M.A., Snipen, L., Lagesen, K., Hallin, P.F., Newell, D.G.,

345 Toszeghy, M., Ridley, A., Manning, G., Ussery, D.W., 2010. Genomic characterization of

346 *Campylobacter jejuni* strain M1. PLoS One 5:e12253

347

348 Gaggia, F., Mattarelli, P., Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe

349 food products. Int. J. Food Microbiol. 141, 15-28.

350

351 Gaggia F., Di Gioia D., Baffoni L., Biavati B., 2011. The role of protective and probiotic cultures in

352 food and feed and their impact in food safety. Trends Food Sci. Tech. 22, S58-S66.

353

354 Gaggia F., Baffoni L., Di Gioia D., Accorsi M., Bosi S., Marotti I., Biavati B., Dinelli G., 2013.

355 Inoculation with effective microorganisms of *Lolium perenne* L.: evaluation of plant growth

356 parameters and endophytic colonization of roots. New Biotechnol. 30, 695-704.

357

358 Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A., Roberfroid, M.B., 2004. Dietary modulation

359 of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17, 259-275.

360

361 Hill, T., Walsh, K., Harris, J., Moffett, B., 2003. Using ecological diversity measures with bacterial

362 communities. FEMS Microbiol. Ecol. 43, 1-11.

363

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

365 T., Wigley, P., 2014. *Campylobacter jejuni* is not merely a commensal in commercial broiler
366 chickens and affects bird welfare. MBio 5, e01364-14.
367

368 ISO. Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for Detection and
369 Enumeration of *Campylobacter* spp. - Part 2: Enumeration Method (ISO 10272:2006).
370

371 Josefsen, M.H., Löfström, C., Hansen, T.B., Christensen, L.S., Olsen, J.E., Hoorfar, J., 2010. Rapid
372 quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and
373 propidium monoazide treatment, as a tool for quantitative risk assessment. Appl. Environ.
374 Microbiol. 76:5097-5104.
375

376 Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrnDB: the ribosomal RNA
377 operon copy number database. Nucleic Acids Res. 29, 181-184.
378

379 Lee, Z.M., Bussema, C. 3rd, Schmidt, T.M., 2009. rrnDB: documenting the number of rRNA and
380 tRNA genes in bacteria and archaea. Nucleic Acids Res. 37, 489-493.
381

382 Mäkeläinen, H., Saarinen, M., Stowell, J., Rautonen, N., Ouwehand, A.C., 2010. Xylo-
383 oligosaccharides enhance the growth of bifidobacteria and *Bifidobacterium lactis* in a simulated
384 colon model. Beneficial Microbes. 1, 81-91.
385

386 Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more
387 out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol. 10, 1571-
388 1581.
389

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

393

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

396

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

400

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

403

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

407

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

410

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

413

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

416

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

420

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

424

425 Sheppard, S.K, Colles, F., Richardson, J., Cody, A.J, Elson, R., Lawson, A., Brick, G., Meldrum,
426 R., Little, C.L., Owen, R.J., Maiden, M.C.J., McCarthy, N.D., 2010. Host association of
427 *Campylobacter* genotypes transcends geographic variation. *Appl. Environ. Microbiol.* 76, 5269-
428 5277.

429

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

432

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

437

438 Wang, J., Baoguo Suna, B., Caoa, Y., Wanga, C., 2010. *In vitro* fermentation of
439 xylooligosaccharides from wheat bran insoluble dietary fiber by bifidobacteria. *Carbohydr. Polym.*
440 82, 419-423.

441

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).

445

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

447

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.

450

451

452

453

454

455

456

457

458

459

460

461

462

463

464

465

466

467

468

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

470

471

472

473

474

475

476

477

478

479

480

481

482

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

		<i>Campylobacter</i> spp.		<i>C. jejuni</i>	<i>Bifidobacterium</i> spp.	<i>B. longum</i>
		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

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

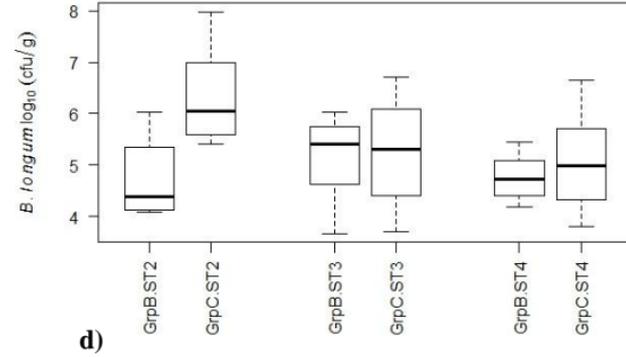
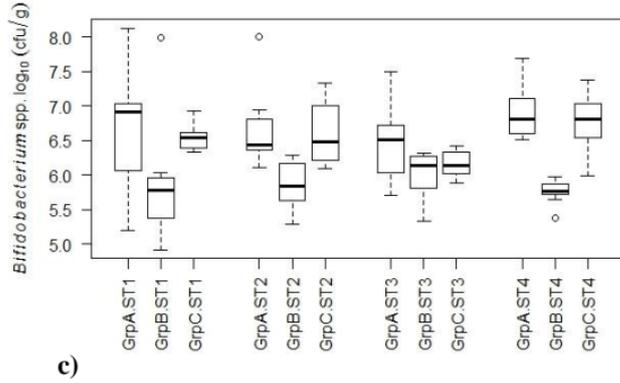
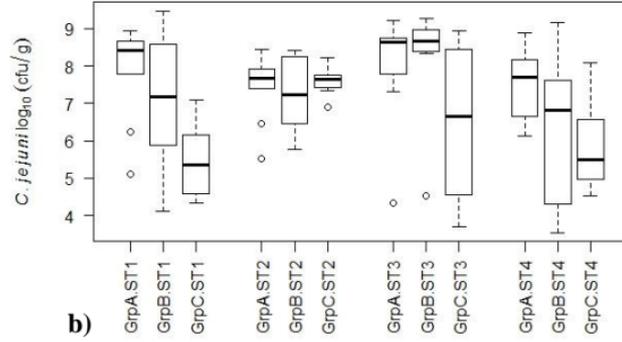
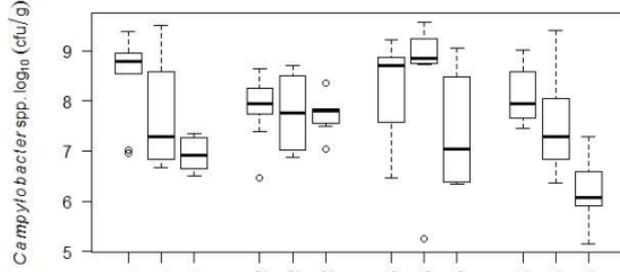
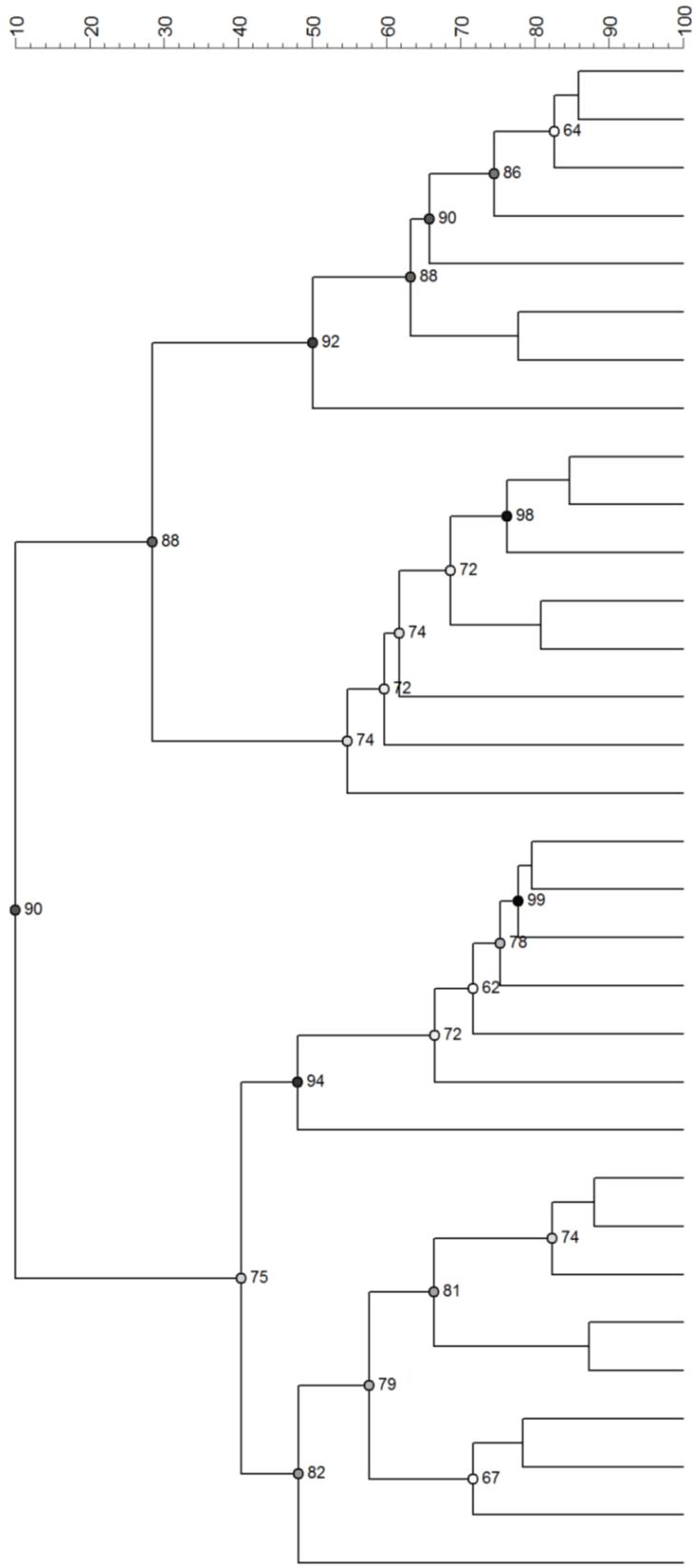
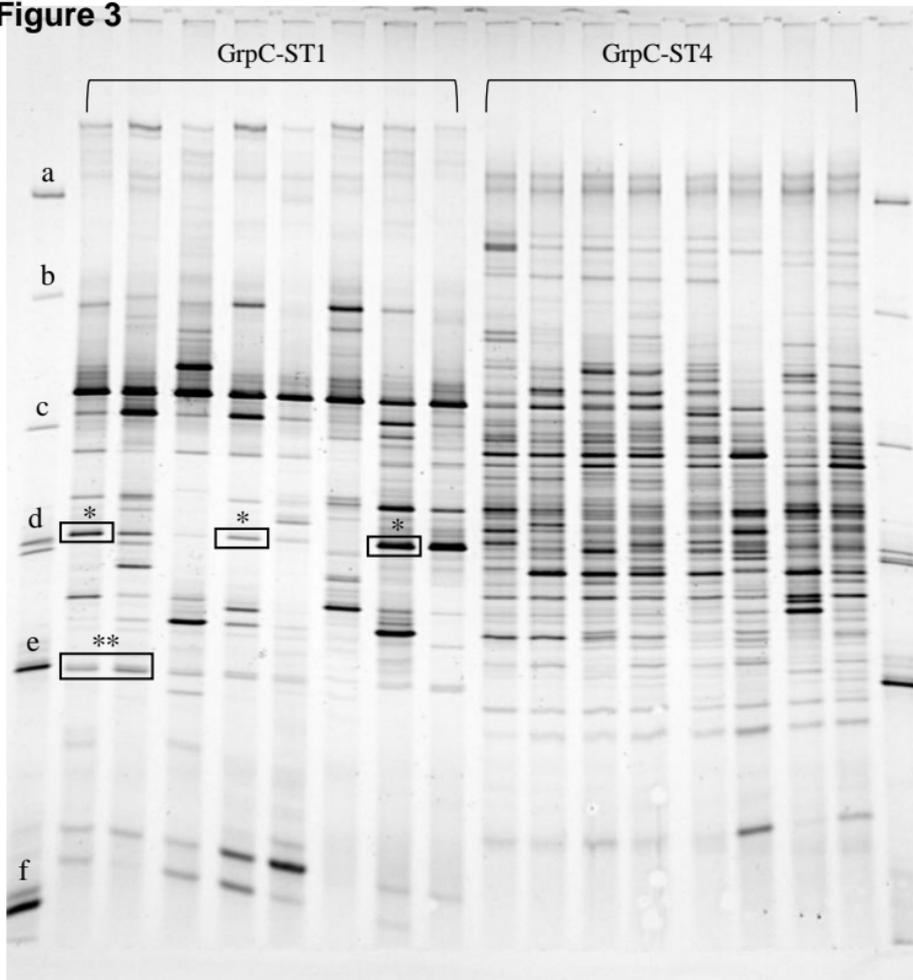
Figure 1

Figure 2



- 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-ST1
- 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
GGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCGACGCCGCG
TGAGGGATGGAGGCCTTCGGGTTG
TAAACCTCTTTTATCGCGGGCAGC
AAGCGAGAGTGAGTTTACCCGT3'
```