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HISTOLOGICAL AND BACTERIOLOGICAL CHANGES IN INTESTINE OF BELUGA (*HUSO HUSO*) FOLLOWING *EX VIVO* EXPOSURE TO BACTERIAL STRAINS

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Abstract

In the present study the intestinal sac method (*ex vivo*) was used to evaluate the interactions between lactic acid bacteria and staphylococci in the gastrointestinal (GI) tract of beluga (*Huso huso*). The distal intestine (DI) of beluga was exposed *ex vivo* to *Staphylococcus aureus, Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Histological changes following bacterial exposure were assessed by light and electron microscopy. Control samples and samples exposed only to *Leu. mesenteroides* and a combination of *Leu. mesenteroides* and *Staph. aureus,* had a similar appearance to intact intestinal mucosal epithelium, with no signs of cellular damage. However, exposure of the DI to *Staph. aureus* and *L. plantarum* resulted in damaged epithelial cells and disorganized microvilli. Furthermore, 16S rDNA PCR denaturing gradient gel electrophoresis (PCR-DGGE) was used to investigate the adherent microbiota of distal beluga intestine. Several bacterial species were identified by DGGE in the present study that have not previously been identified in beluga.

Key words: Beluga, intestine, bacteria, light and electron microscopy, DGGE

1. **Introduction**

The gastrointestinal (GI) tract of fish is thought to be an important portal for bacterial infection and it has been reported that an essential and prerequisite step for bacterial invasion is the translocation of bacteria across the intestine; however this is difficult to study effectively *in vivo* and such studies are time consuming and have high cost. According to EU regulations, it is recommend that effort be focused on reducing the numbers of *in vivo* experiments and numbers of experimental fishes (Revision of the EU directive for the protection of animals used for scientific purposes [Directive 86/609/EEC]; 08th of September 2010). In recent years three different *ex vivo* methods (the Ussing chamber, everted sack and intestinal sack) have been used in order to fulfil the instructions given by EU. These methods have been used to evaluate translocation and cell damage caused by pathogenic bacteria (Ringø et al., 2004; Ringø et al., 2007 a; 2007 b; Jutfelt et al., 2008; Salinas et al., 2008; Khemiss et al., 2009; Ringø et al., 2010; Løvmo Martinsen et al., 2011). To the author's knowledge, no information is available from sturgeon studies on morphological changes and cellular responses in the gut epithelium after *ex vivo* exposure to lactic acid bacteria or a staphylococci. Therefore the 1st objective of the present investigation was to evaluate by means of light and electron microscopy whether exposure of the distal intestine (DI) of the beluga (*Huso huso*) to a *Staphylococcus aureus* originally isolated from the gastrointestinal (GI) tract of beluga affects the morphology of the intestinal epithelium in the DI. Furthermore, we also want to evaluate whether there are different responses when DIs are exposed to only sterile saline (no bacteria), *Leuconostoc mesenteroides* originally isolated from DI of Persian sturgeon (*Acipenser persicus*) and two combinations of *Staph. aureus* and *Leu.*

65 *mesenteroides*. As some authors have hinted on the use of lactic acid bacteria isolated
66 from other sources than fish as probionts in aquaculture (Bagheri et al., 2008; Salinas et
67 al., 2008; Merrifield et al., 2010a), the 2nd aim of the present study was therefore to assess
68 whether a *Lactobacillus plantarum* originally isolated from Sabalan cheese was able to
69 adhere to and colonise the distal part of beluga intestine.
70 As some investigations have focused on antagonistic effect of different strains of lactic
71 acid bacteria towards *Vibrio* (*Listonella*) *anguillarum* and *A. salmonicida* (Ringø et al.,
72 2005; Ringø, 2008) we would like to investigate the interactions of an indigenous species
73 (*Staph. aureus*) and *Leu. mesenteroides* as the latter strain might be a potential probiotics
74 in future sturgeon aquaculture **(**Askarian et al., 2011).
75 To our knowledge, only two studies have presented information on bacteria communities
76 in studies using the intestinal sack method (Ringø et al., 2010; Løvmo Martinsen et al.,
77 2011). However, these studies utilised culture-based techniques, which are time
78 consuming and do not present a correct picture regarding the microbial diversity even if
79 several different media are used (Amann et al., 1995; Asfie et al., 2003; Zhou et al.,
80 2007). Therefore, in order to present more reliable information to identify and quantify
81 intestinal microbiota of fish, molecular method such as Polymerase Chain Reaction-
82 Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (method described by Muyzer et
83 al. 1993) has been used (Zhou et al., 2007; Liu et al., 2008; Zhou et al., 2009 a; 2009 b).
84 DGGE remains the method of choice due to its rapid, sensitive and inexpensive nature
85 when assessing the gut microbiota of fish (Hovda et al., 2007; Zhou et al., 2007; Liu et al.,
86 2008; Zhou et al., 2009 a; 2009 b). This method provides information of the dominant
87 bacteria by excising bands from the DGGE gel, followed by reamplification and

sequencing (Liu et al., 2008). The 3rd aim of the present study was therefore to assess the adherent microbial community by DGGE in *ex vivo* studies where the distal beluga intestines were exposed to sterile saline solution and different treatments of bacteria.

In the present study, live bacteria was used as there are indications that exposure of the intestinal epithelium to live pathogenic bacteria create epithelial damage and shedding of enterocytes (Ringø et al., 2004) and that bacterial translocation rates are significantly higher when using live pathogens compared to heat-inactivated bacteria (Jutfelt et al., 2008). Furthermore, live bacteria were used in order to evaluate whether they were able to colonise the DI.

2. Materials and methods

2.1. *Fish*

In the present *ex vivo* experiment 16 unvaccinated beluga (*Huso huso*) were used, 15 fish for 5 treatments (3×5 treatments) and 1 fish for 6th treatment. The main reason for using only one fish in the 6th treatment was due to shortage of fish. All the fish were reared at Shahid Rajaie Sturgeon Centre, Sari, Mazandaran, Iran and fed a commercial diet (45% protein, 14% fat and 10% carbohydrates (Chine Co., Iran), at 4% of their body weight per day. The water temperature was 10°C, and the water flow was 1 l/min. Fish were adapted to the experimental conditions for 12 hours before the initiation of the *ex vivo* experiments. At the time of sampling the fish had average weight between 250-350g.

2.2. *Bacteria*

Bacteria used in the *ex vivo* study were *Staphylococcus aureus* and *Leuconostoc mesenteroides*. The *Staph. aureus* strain identified by 16S rRNA gene sequencing according to Ringø et al. (2006) showed 99% similarity to *Staph*. *aureus* ssp. *aureus* (GenBank accession no. CP000730) and was autochthonous strain originally isolated from distal intestine (DI) of beluga (Askarian and Ringø, unpublished data). *Staph*. *aureus* was grown on tryptic soya agar (Riedel –Dehaen, Germany) plates supplemented with 5 % glucose and 1 % NaCl (TSAgs) at 30^{o}C for 2 days. Prior to *ex vivo* exposure, the bacteria were grown in tryptic soya broth for 24 h. In order to estimate the exact numbers of bacteria, the optical density at 600 nm (OD_{600}) was measured and counts were conducted from serial dilutions of the bacterial suspensions onto TSAgs plates. In our *ex vivo* experiment we used 7.4 x 10^6 bacteria per ml. When the DI of beluga was

120 only exposed to *Staph. aureus*. When *Staph. aureus* was used in combination with *Leu.*
121 *mesenteroides*, the count of the staphylococci strain was 2.6×10^{6} CFU per ml. 16S
122 rRNA gene sequencing analysis of the *Leu. mesenteroides* strain showed 98 % similarity
123 to *Leu. mesenteroides* NRIC 1517 (GenBank accession no. AB362705) (Ringø,
124 unpublished data). The bacterial strain was autochthonous originally isolated from the DI
125 of Persian sturgeon (Askarian et al., 2009). Prior to *ex vivo* exposure, *Leu. mesenteroides*
126 were grown in marine broth (Difco™, 2216 Marine broth, USA) for 2 days. The OD_{600}
127 was measured and counts were conducted from serial dilutions of the bacterial
128 suspensions onto marine agar plates (4.1×10^{6} CFU per ml). In addition a lactobacilli
129 strain originally isolated from traditional Sabalan Iranian cheese from sheep raw milk
130 was used. The strain was kept for 3 months in salt water before use in the present study.
131 The lactobacilli strain was identified by 16S rRNA gene sequencing and showed 98%
132 similarities to *Lactobacillus plantarum* (GenBank accession no GQ423760) (Tajabady,
133 unpublished data). *L. plantarum* was cultured in MRS broth (Fluca, catalogue no. 69966)
134 and incubated under anaerobic conditions at 37°C for 24h before *ex vivo* exposure. The
135 OD_{600} was measured and counts were conducted from serial dilutions of the bacterial
136 suspensions on to marine agar plates (8.6×10^{6} CFU per ml).
137
138 2.3. *Ex vivo exposure to bacteria*
139 Fish were killed by a blow to the head. The intestine (from just posterior to the
140 attachment of the pyloric caeca to the anus) was carefully removed and the intestinal
141 contents were gently emptied and thoroughly rinsed three times with sterile 0.9 % saline
142 solution. Intestines were tightly tied at the distal end and filled up with approximately

143 1 ml 0.9 % sterile saline solution or bacterial suspensions as described in Table 1.
144 Thereafter the proximal end was immediately tied and placed into sterile falcon tubes
145 containing sterile saline solution. The gut samples were incubated at 10°C in a cooling
146 bath. For detailed information of the experimental treatments see Table 1. After
147 incubation the intestine was cut free at the both ends and rinsed 3 times with sterile saline
148 solution and a 0.5 cm segment from the distal part of the intestine was excised for
149 histological evaluation. The remaining part of the intestine was immediately fixed in 96
150 % ethanol for denaturing gradient gel electrophoresis (DGGE) analysis.
151

152 2.4. *Microscopic sampling*

153 Distal intestinal segments from each treatment group were immediately fixed in
154 McDowell's fixative (McDowell and Trump, 1976) and prepared for analyses by light
155 microscopy (LM), transmission electron microscopy (TEM), and scanning electron
156 microscopy (SEM).

157 Gut samples for LM were washed 2 times in Sørensen's buffer and then postfixed in
158 OsO_4. After serial dehydration steps in alcohol (30% - 100%), samples were placed in 1,
159 2-propylenoxide prior to being embedded in agar 100 Resin. One µm thick sections were
160 stained with 2% toluidine blue and examined under light microscope (Leica DMLB)
161 Images were acquired by means of a Leica DC 300 digital camera.

162 Preparation of gut samples for TEM and SEM was performed as described elsewhere
163 (Ringø et al., 2001). Samples were examined under transmission microscope (JEM-1230).
164 Samples for SEM were washed 2 times in Sørensen's buffer and then postfixed in OsO_4

165 (1% in Sørensen's buffer, 2 h). After serial dehydration steps in alcohol (30% to 100%),
166 samples were critical-point dried (BALZERS CPD 020), after that mounted on aluminum
167 stubs, sputter-coated with gold by using a high-resolution fine coater (SC7640,Quorum
168 technologies Ltd,UK), and examined under a JSM-6300 scanning microscope.

169 In order to determine morphological differences in the DI of beluga of the different
170 treatments, 10 randomly selected samples from each fish in each treatment group were
171 taken for LM and TEM images. The effects of treatments were monitored in terms of the
172 presence of cell debris in the lumen, disorganized microvillus, budding from the apices of
173 microvillus, edema, and disintegrated tight junctions, dark cellular bodies under lamina
174 propria, loss of epithelial integrity, goblet cells and lysozyme. Differences were ranked as
175 follows according to Ringø et al. (2007 b); 0 = not observed, 1 = low (1–3 out of 10
176 images), 2 = moderate (4–6 out of 10 images), and 3 = high (7 or more out of 10
177 images) frequency of occurrence.

178

179 2.5. *In vitro growth inhibition of Staph. aureus by Leu. mesenteroides*

180 *In vitro* growth inhibition was tested using microtitre plate assay as described elsewhere
181 (Ringø et al., 2005; Ringø, 2008). Growth of *Leu. mesenteroides* and *Staph. aureus* were
182 examined by direct (total viable counts) and indirect measurements (optical density;
183 OD_{600}). Both bacterial strains were grown on tryptic soy broth media supplemented with
184 1% glucose (TSBg).

185 *Leu. mesenteroides* and *Staph. aureus* were pre-cultured in 4.5 ml sterile Eppendorf tubes
186 containing TSBg medium and the bacteria were harvested in the stationary growth phase.

187 *Leu. mesenteroides* was centrifuged at 4000 rpm for 20 minutes, and the supernatant was
188 sterile-filtered (Filter Syringe 0.2 µm, Acrodisc®). Prior to the microtitre plate assay 20 µl
189 of bacterial suspensionon of *Staph. aureus* was diluted in 5ml TSBg, kept for 2 hours at
190 21°C and OD_{600was} measured. In order to use appropriate concentration of bacteria of this
191 bacterial suspension to (OD_{600}= 0.05/0.06; corresponded to 5-6 $\times 10^7$ bacteria/ml) 3-20 µl
192 were transferred to 10ml sterile broth media with and without 1 % supplementation of
193 NaCl. Each microtiter plate well contained 50 µl of the sterile supernatant of *Leu.*
194 *mesenteroides* and 50 µl bacterial suspension of *Staph. aureus.* Sterile growth media and
195 *Staph. aureus* was used as a positive control. Growth was estimated at OD_{600nm} and was
196 carried out at 30°C. Measurements were carried out each hour using an automatic plate
197 reader, Bioscreen C (Labsystems, Finland).
198
199 2.6. *DNA extraction and PCR amplification*

200 Genomic DNA was obtained using the extraction method described by Brady (2007) with
201 some modifications. Briefly, 200µl sample was transferred to a 1.5ml Eppendorf tube and
202 centrifuged at 12,000 rpm for 10min. The supernatant was discarded and the pellet air-dry
203 at 25°C. 700 µl lysis buffer was added to each tube and mixed thoroughly. Both samples
204 were incubated for 4h in a 70°C water bath. Each bottle was gently inverted every 30min.
205 Post incubation the samples were centrifuged at 10,000rpm for 18min. The supernatant
206 was poured into a clean tube and an equal volume of isopropanol was added and gently
207 mixed by inversion prior to incubation at -20°C for 30min. After incubation the samples
208 were centrifuged at 12,000 rpm for 15 min and the supernatant discarded. The liquid was

209 aspirated off the pellet. The pellet was resuspended in 50μL TE. Genomic DNA was
210 thereafter purified using a agarose gel DNA purification kit (TaKaRa, Dalian, China).

211 The V3 region of the rrs gene was amplified. The primer and PCR reaction system is as
212 described elsewhere (Liu et al., 2008; Zhou et al., 2009 a). Amplification consisted of an
213 initial denaturation step at 95^{o}C for 5 min, followed by 28 cycles at 94^{o}C for 30s at 56^{o}C
214 for 30s and a final extension at 72^{o}C for 30s. An additional final extension at 72^{o}C for 7
215 min was used.

216 2.7.*Denaturing Gradient Gel Electrophoresis (DGGE) analysis*

217 DGGE was performed as described elsewhere (Liu et al., 2008; Zhou et al., 2009 a).
218 Electrophoresis was conducted with a constant voltage of 80V at 61^{o}C for about 14h.
219 Gels were stained with ethidium bromide for 20 min, and photographed with UV
220 transillumination.

221 Computer-assisted comparison of DGGE patterns was performed with BIO-ID++ gel
222 analysis software (Vilber-Lourmat, Torcy, France). Relative abundance (RA, %) was
223 represented by percentage of a specific band intensity to the total band intensity. Cluster
224 analysis was based on the unweighted pair group method using the airthmetic mean
225 algorithm (UPGMA). In this study, similarity coefficients (Cs)<0.60 is regarded as
226 significant difference, 0.60≤Cs<0.85 as marginal difference and Cs ≥0.85 is treated as
227 similar. Relative abundance (%) was represented by the percentage of a specific band
228 intensity to the total band intensity (Zhou et al., 2009 b). The Shannon diversity index H
229 = $-\sum RA_i \ln(RA_i)$ and Shannon equitability index $E_H = H/\ln(S)$ (where RA_i is the proportion

of the *i*th band and *S* is the total number of visual bands) were calculated using spreadsheet software (V0.1, Microsoft Inc., CA, USA) (Dethlefsen et al., 2008).

2.8. *Sequencing of the 16S rDNA*

The DNA fragments selected for sequencing were excised and amplified using the primers 338f without GC clamp and 519r following the procedure of Liu et al. (2008). All sequences were submitted for similarity searches with the BLAST program, and deposited in the NCBI database under GenBank accession numbers GU301183-GU301249.

3. **Results**

Overviews of the different morphological changes observed in the six treatments are presented in Table 2 (light microscopy; LM) and Table 3 (transmission electron microscopy; TEM). Evaluation of the epithelial cells exposed to only sterile saline solution (treatment 1) by LM (Table 2) and TEM (Table 3) showed intact epithelium with lamina propria, undamaged enterocytes, well organized microvilli (MV), lots of nuclei and normal looking mucosa with an intact epithelium. Similar results were obtained from LM and TEM when the distal intestines (DIs) were exposed to *Leuconostoc mesenteroides* (treatment 3) (Table 2 and 3). However, DIs first exposed to *Leu. mesenteroides*, rinsed 3 times with sterile saline and thereafter exposed to *Staphylococcus aureus* ssp. *aureus*. (*Staph*. *aureus*) (treatment 5) showed almost similar morphological features as the control group (Table 2 and 3). Although some TEM micrographs (3 out of 10) showed disorganised MV and the apical part of enterocyte displayed loss of MV, no clear sign of cell damage was observed.

Scanning electron microscopy (SEM) micrographs of the DI first exposed to *Leu. mesenteroides* and thereafter to *Staph*. *aureus* (treatment 5) (Figure 1) showed uniform organised microvilli and various lengths of enterocytes. Similar results were observed when the DIs were exposed to only sterile saline solution (results not shown) or only *Leu. mesenteroides* (results not shown). In contrast to these results, exposure of DIs first to *Staph*. *aureus* and thereafter to *Leu. mesenteroides* (treatment 6) resulted in clear changes in the intestinal epithelium (Figure 2). Serious signs of tissue damage included cell debris in the lumen, affected (long) microvilli in some areas of the epithelium, protruding cells and disintegrated tight junctions, numerous numbers of intra epithelial lymphocyte – like

cells were noted. Similar results from LM and TEM evaluations were also obtained when the DI was exposed to only *Staph. aureus* (treatment 2) (results not shown). SEM micrographs showed extensive loss of microvilli when the DIs were incubated with only *Staph*. *aureus* (treatment 2) or exposure first to *Staph*. *aureus* and thereafter exposed to *Leu. mesenteroides* (treatment 6) (results not shown). Furthermore, DIs exposed to *Lactobacillus plantarum* (treatment 4) displayed extensive cell damage; loss of microvilli, damage of intracellular tight junctions, disintegrated microvilli and dense chromatin (Table 2, 3 and Figure 3).

Figure 4 shows growth of *Staph*. *aureus* measured by optical density (OD_{600}) and maximum OD_{600} value (0.65) of bacteria, the control group was obtained approximately after 45 hours. However, growth of *Staph*. *aureus* was inhibited (OD_{600} = 0.35) when the incubated with *Leu. mesenteroides* supernatant.

Denaturing Gradient Gel Electrophoresis (DGGE) fingerprint analysis of the bacterial communities, representatives of the adherent bacteria, and their relative abundance (RA; %) are showed in Table 4. Cluster analysis of the band patterns are displayed in Figure 5, and the pairwise similarity coefficients (C_s) matrixes of the adherent bacterial community are showed in Table 5.

Samples no. 101, 102 and 103 are from the control group (exposed to sterile saline), while no. 104, 106 and 107, no. 108, 109 and 110, no. 111, 112 and 113, no. 118, 119 and 120, and no. 121 are samples taken from DI exposed to *Stap*. *aureus* (treatment 2), *Leu*. *mesenteroides* (treatment 3), *L*. *plantarum* (treatment 4), *Leu*. *mesenteroides* and *Stap*. *aureus* (treatment 5) and *Stap*. *aureus* and *Leu*. *mesenteroides* (treatment 6), respectively.

286 An average of twelve bands were identified in the control group while 17.33, 13.67, 5.00,
287 8.00 and 9.00 species were identified in treatment 2, 3, 4, 5 and 6, respectively (Table 4).
288 Compared to the control, the relative abundance of 5 species were significantly lower
289 ($P<0.05$) in treatment 2, while 6 species were elevated ($P<0.05$) in treatment 2. As well as
290 treatment 3 was concerned, 8 species were lower ($P<0.05$) compared to the control,
291 however, 6 species were elevated ($P<0.05$). For treatment 4, 9 species were lower
292 ($P<0.05$) compared to the control, however, 3 species were elevated ($P<0.05$). In
293 treatment 5, 8 species were lower ($P<0.05$) compared to the control, however, 1 species
294 were elevated ($P<0.05$). Compared to the control, 8 species seem to be lower; however, 9
295 species seem to be elevated in treatment 6.
296 *Plesiomonas shigelloides* - like, *Shewanella* sp. – like, uncultured *Shewanellaceae*
297 bacterium - like and *Aeromonas* sp. were significantly lower ($P<0.05$) in all treatments
298 compared to the control. Uncultured bacterium-like (GenBank accession no. GQ468111)
299 was elevated ($P<0.05$) in treatment 6, however, this species was lower in treatment 3 and
300 4 ($P<0.05$).
301 In the present study we were not able to identify bands showing similarity to *Leu*.
302 *mesenteroides* in the treatments (3, 5 and 6) exposed to *Leu*. *mesenteroides*. On the other
303 hand, the uncultured *Staphylococcus* sp. - like bacterium (band B21) identified in
304 treatment 5 and 6 showed 99 % similarity to *Stap*. *aureus* GenBank accession no.
305 CP000730 used in the present study. Furthermore, four bands (B6, B7, B8 and B11) in
306 treatment 4 showed high (99 %) similarity to *L*. *plantarum* used in the present study.
307 Based on the cluster analysis, the microbial patterns of the experimental treatments
308 showed clear differences to that of the control, in which treatment 5 (C_s=0.36) showed

309 the maximal difference to the control and treatment 3 showed the minimal difference
310 (C_s=0.56) to the control. The C_s of treatment 5, 2, and 4 to the control was 0.47, 0.50, and
311 0.53 respectively (Table 5). The microbial patterns between treatment 5 and 6 showed
312 very similar (C_s=0.89).
313

4. Discussion

To our knowledge no information is available on morphological changes and cellular responses and occurring in the gut epithelium of beluga (*Huso huso*) after *ex vivo* exposure to "good" or indigenous bacteria. The *ex vivo* approach used in the current investigation may be useful for evaluating bacteria-induced morphological changes in intestinal epithelium under controlled experimental conditions and can be valuable approach for reducing the number of fish to a minimum in studies evaluating the interactions between bacteria (Salinas et al., 2008). The DI of beluga exposed to bacteria strains revealed apparent differences between *Staph. aureus* and *Leu. mesenteroides*. These effects were clearly observed by LM, TEM and SEM, where epithelial cells with disorganized and altered microvilli, damaged tight junctions, protruding epithelial cells sloughing into the lumen, and numerous intraepithelial lymphocyte-like cells in groups treatmented with *Staph. aureus*. As there is no evidence of staphylococci induced effects on beluga gut histology, the results of the present study are of importance with respect to whether the intestine is involved in the pathogenesis of *Staph. aureus*. Changes in the number of goblet cells and immune cells (intra epithelial like cells) were also observed when the DI was exposed to bacteria. DI exposed to *Leu. mesenteroides* was histological similar to control samples showing an intact epithelial barrier, which confirms the previous results on Atlantic salmon where indigenous bacteria do not affect gut cellular integrity (Ringø et al., 2004). When the DI was first exposed to *Leu. mesenteroides* and subsequently exposed to *Staph. aureus* the intestinal morphology was more or less similar to the finding of the control group or the treatment group only exposed to *L. mesenteroides*. Some degrees of epithelial changes were observed but no clear cell

damages were noticed. Based on our results we suggest that *Leu. mesenteroides*, is able to prevent, to some extent, intestinal induced damage caused by *Staph. aureus* in the DI of beluga. As severe cell damage was observed by exposure of the DI first to *Staph. aureus* and thereafter to *Leu. mesenteroides*, it seems that non-pathogenic bacteria do not reduce the tissue damaging effects where most of the cell damage occurred by *Staph. aureus*. Similar results were obtained when the DI was only exposed to *Staph. aureus*. Based on our results we suggest that the DI might be an important infection route for *Staph. aureus* in beluga, but information is lacking whether the proximal intestine of beluga is involved in *Staph. aureus* infection in the fish. This topic merits further investigation.

Several authors have suggested that *Lactobacillus* species isolated from other sources than fish might be good candidates as probiotics in fish (Bagheri et al., 2008; Salinas et al., 2008; Merrifield et al., 2010a). However, as the present study clearly demonstrated that a *Lactobacillus plantarum* originally isolated from Iranian cheese caused severe cell damage in the DI of beluga we conclude that light and electron microscopy evaluations should be included as an important criteria in future selection of *Lactobacillus* species as probiotics in beluga.

Several "new" bacterial species were identified in the present study that have not previously been described in the GI tract of beluga. We suggest that these bacteria probably belong to the autochthonous gut microbiota as the DI was rinsed several times prior to sampling. Some investigations have been published on the presence of allochthonous *Escherichia coli* in the GI tract of fish (Jiang et al., 2009; Liu et al., 2009; Tang et al., 2009; Feng et al., 2010). However, to our knowledge the present study is the

first one detecting autochthonous *E. coli* in the digestive tract of fish and the strain detected in the present study showed 100 % similarity to a *E. coli* previously described by Jeong et al. (2009).

Fresh water fish like tilapia (*Oreochromis mossambicus*) and striped bass (*Morone saxatilis*) are suggested to be the primary reservoir of *Plesiomonas shigelloides* (Nedoluha and Westhoff, 1995, Moreno et al., 2006). In the present study, we detect *P. shigelloides* from two treatment groups that showed high similarity to a *P. shigelloides* previously described by Sarah et al. (unpublished results, National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/).

Two recent investigations have reported that *Shewanella* appeared to be the most abundant bacterium in the digestive tract of Atlantic salmon (*Salmo salar*) (Navarrete et al., 2009) and flounder (*Platichthys flesus*) (Ziolkowska-Klinkoz et al., 2009). Our DGGE analysis showed that seven bands belonging to different *Shewanella* species in beluga intestine.

In the present study, three bands showed high similarity to *Aeromonas allosaccharophila* previously reported in a study investigating microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*) (Wu et al., 2010). Two bands identified in DI of treatment group 5 showed 100 % similarity to *Aeromonas* sp. previously isolated by Barberio et al. (2001). Li, A. and Yang, W (unpublished results, NCBI) described *Aeromonas media* isolated from water and intestine of silver carp (*Hypophthalmichthys molitrix*). In the present study, 4 bands showed high similarity to *A. media*. Several studies have isolated *Acinetobacter* from fish intestine (Ringø et al., 1995; Ringø and Birkbeck, 1999; Bakke-Mckellep et al., 2007; Hovda et al., 2007). In the

present study we detected one band that showed high similarity to *Acinetobacter* sp. previously described by Davolos and Pietrangeli (2009).

Previous results from DGGE analysis of the microbial community in fish gut has revealed that uncultured bacteria constitute of a major part (He et al., 2009; Zhou et al., 2009a; 2009b; Merrifield et al., 2010b). In our study, 3 bands showed high similarity to two uncultured bacterium clones.

As *Leu*. *mesenteroides* originally isolated from Persian sturgeon (*Acipenser persicus*) was not identified by DGGE in the present study this may indicate that the bacteria was not able to adhere the distal intestinal mucosa of beluga, or the bacteria had translocate into the lamina propria. However, no bacteria were observed in the lamina propria. Interestingly, the present results clearly showed lack of adherence of *Leu*. *mesenteroides* originally isolated from the GI tract of Persian sturgeon to beluga intestine. These results are in accordance with the results of Askarian et al. (2011). In contrast to the results of *Leu*. *mesenteroides*, we put forward the hypothesis that *Staph*. *aureus* originally isolated from beluga and *L*. *plantarum* originally isolated from Iranian cheese were able to colonise the DI of beluga as bands corresponding to these bacteria were detected by DGGE analysis even after thorough rinsing of the intestine. However, we will not recommend the use of *L*. *plantarum* originally isolated from Iranian cheese as a probiont in beluga as the bacteria caused severe cell damage in the DI of beluga.

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Legends to Figures

Figure 1.

Scanning electron microscopy micrograph of the distal intestine (DI) of beluga first exposed to *Leu*. *mesenteroides* (22.5 minutes), rinsed 3 × with sterile saline and thereafter exposed to *Staph*. *aureus* (22.5minutes). The micrograph shows normal looking enterocytes (E) with well organised microvilli (MV). LP - lamina propria (LP); lumen - L. Bar-5µm.

Figure 2.

TEM micrograph of the DI of beluga first exposed to *Staph*. *aureus*, rinsed 3 × with sterile saline and thereafter exposed to *Leu*. *mesenteroides*. The micrograph shows severe cell damage and all the epithelium is absent. LP - lamina propria; BV - blood vessels. Bar - 5µm.

Figure 3.

TEM micrograph of the DI of beluga exposed to *Lactobacillus plantarum* for 45 minutes. The micrograph shows damage endothelial cells with disorganised microvilli (DMV), intra - cytoplasmic vacuoles (V) and signs of oedema (O). Notice also the dense chromatin (C) in the enterocyte nucleus. Bar - 5 µm

Figure 4.

In vitro growth inhibition *of Staph*. *aureus by Leu*. *mesenteroides* optical density (OD_{600}).

Figure 5.

The dendrogram of the PCR-DGGE fingerprints of the V3 region gene of 16S rDNA of the bacteria from the distal intestine of beluga after treated with various bacteria. Samples no; 101, 102 and 103 (treatment 1), 104, 106 and 107 (treatment 2), 108, 109 and 110 (treatment 3), 111, 112 and 113 (treatment 4), 118, 119 and 120 (treatment 5) and 121 from treatment 6.

Figure 1.

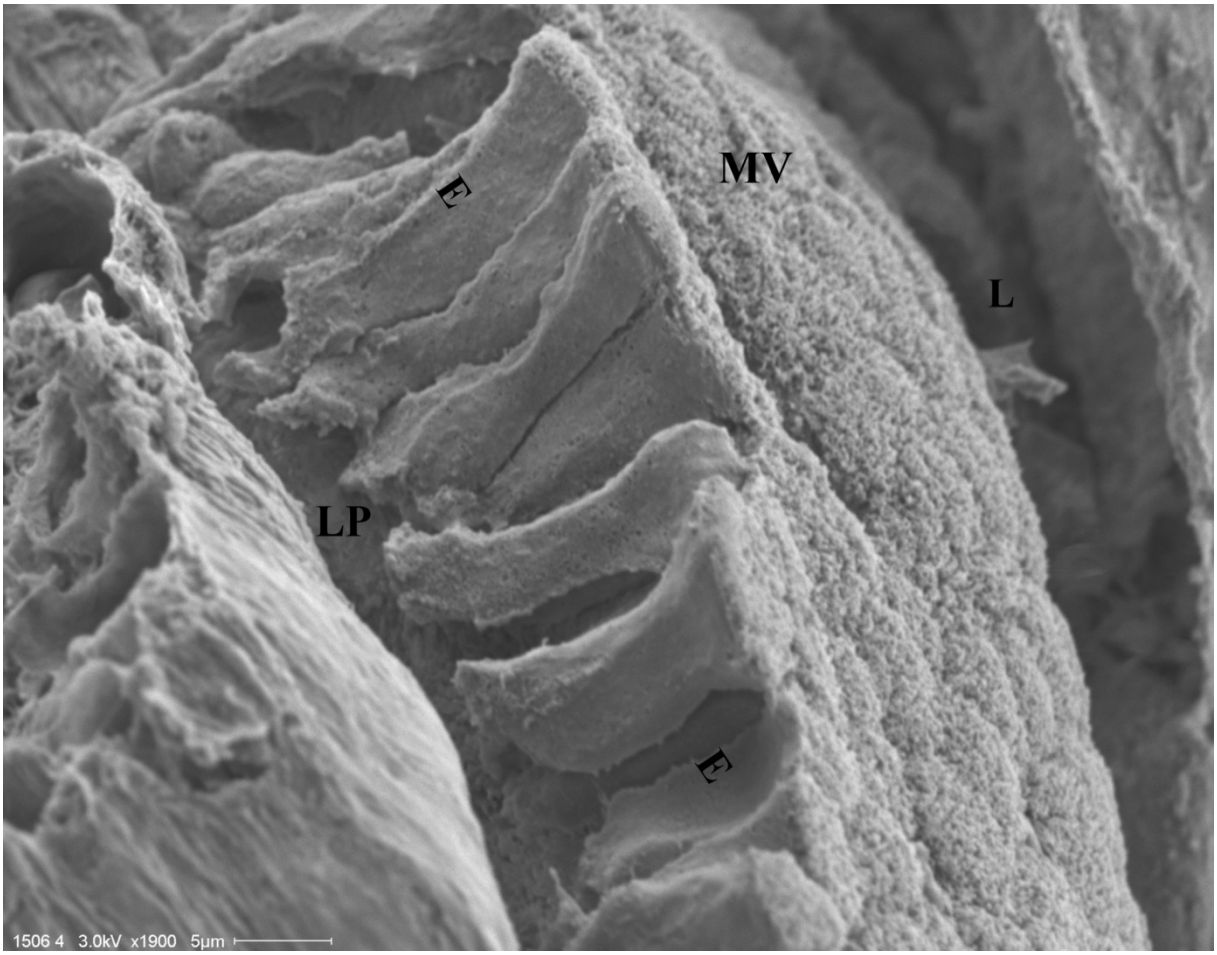
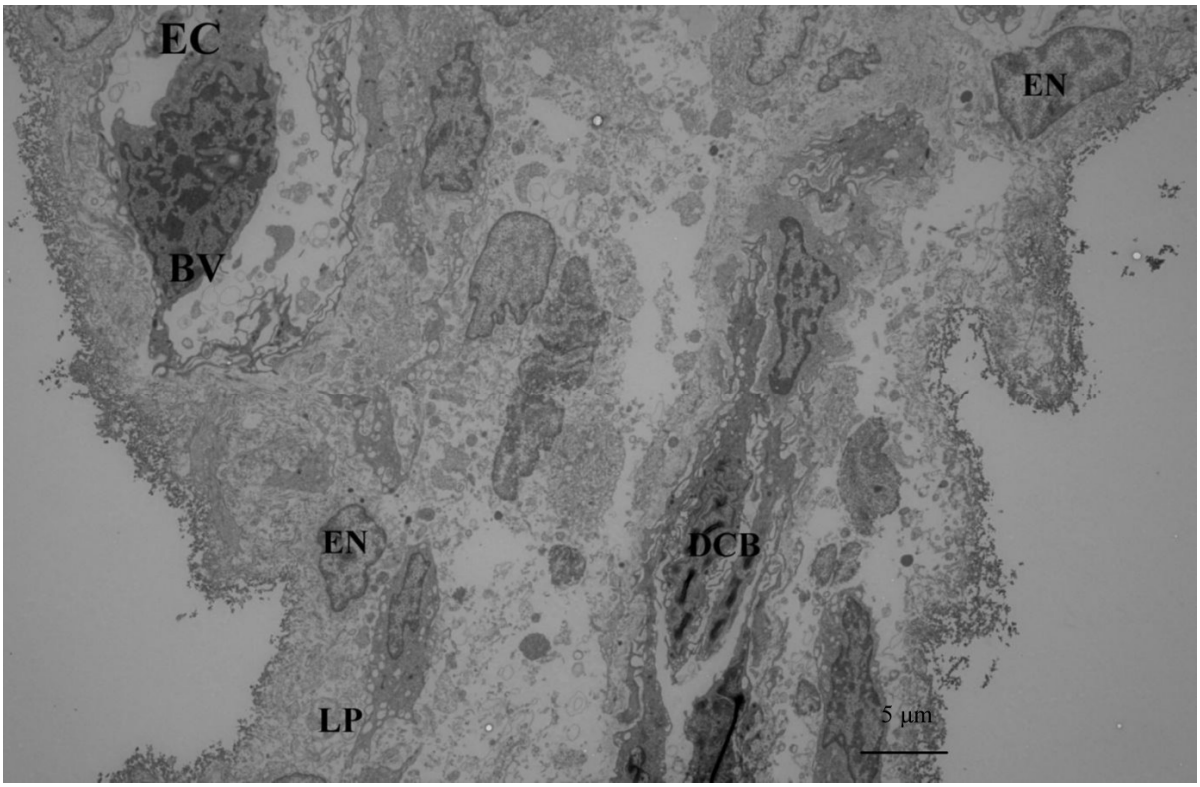


Figure 2.



53 Figure 3.

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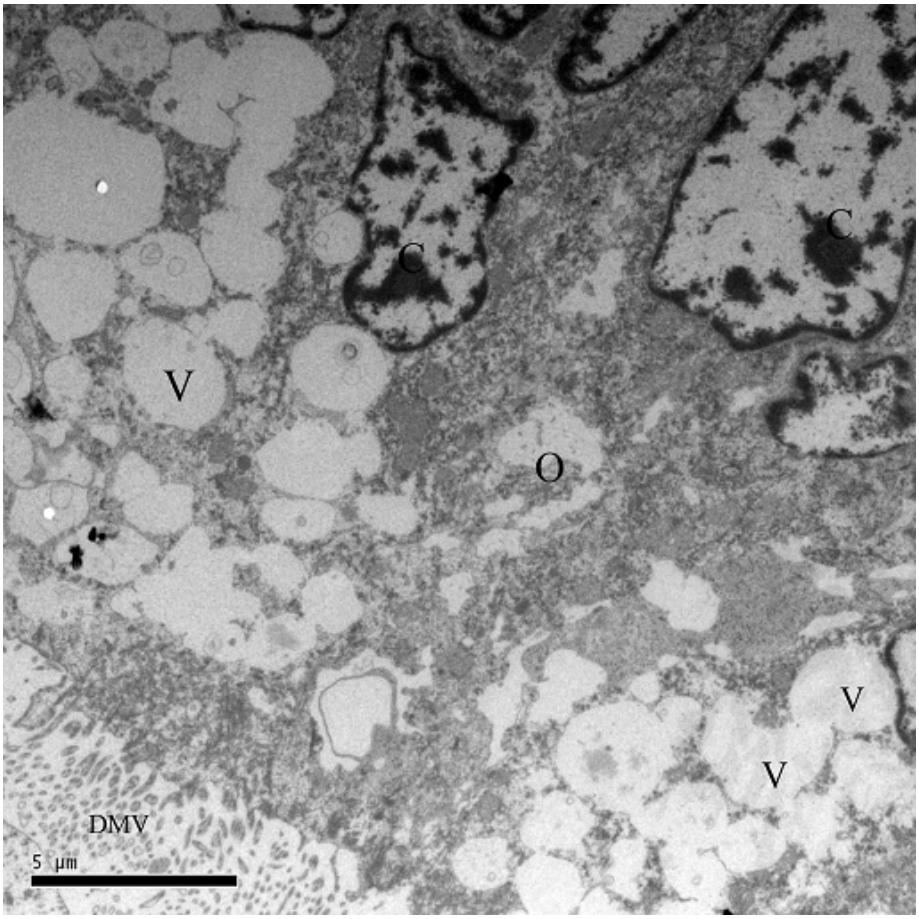

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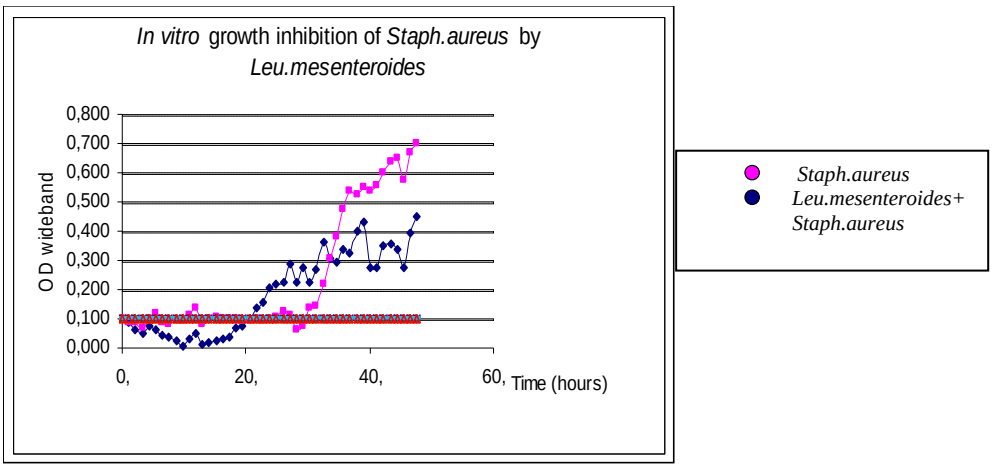
Figure 4.


In vitro growth inhibition of Staph.aureus by Leu.mesenteroides
OD wideband
0,800
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40,
60,
Time (hours)
Staph.aureus
Leu.mesenteroides+ Staph.aureus

61 Figure 5.

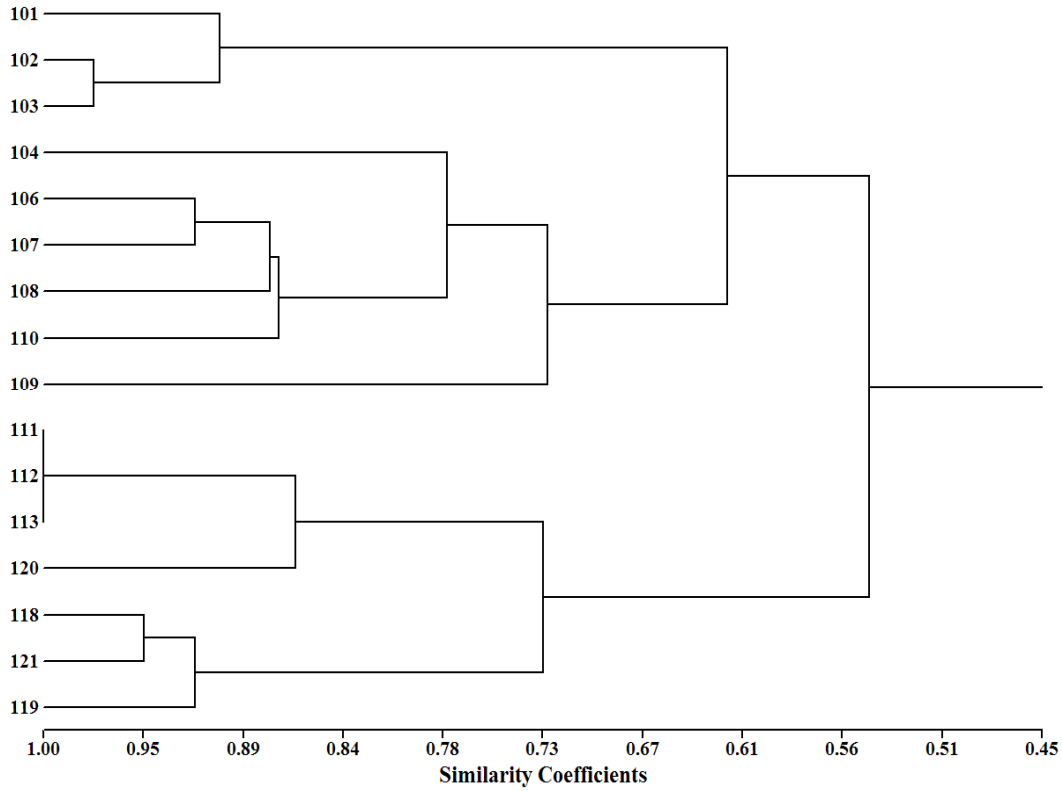

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Table 1. Experimental treatments applied to beluga (*Huso huso*) during *ex vivo* exposure to various bacterial strains. Three fish were used in treatment 1 to 5 while only one fish were used in treatment 6.

Treatment	Bacterial strain and dose (CFU/ml)	Exposure time	Rinsed*	Exposure time	Rinsed*
1	Sterile physiological saline	45 min	Yes		
2	*Staphylococcus aureus* spp. *aureus* (7.4×10^6)	45 min	Yes		
3	*Leuconostoc mesenteroides* (4.1×10^6)	45 min	Yes		
4	*Lactobacillus plantarum* (8.6×10^6)	45 min	Yes		
5**	*L. mesenteroides* (4.1×10^6) *Staph. aureus* spp. *aureus* (2.6×10^6)	22.5 min –	Yes	– 22.5 min	Yes
6***	*Staph. aureus* spp. *aureus* (2.6×10^6) *L. mesenteroides* (4.1×10^6)	22.5 min –	Yes	– 22.5 min	Yes

CFU – colony forming units; * - prior to sampling the distal intestine (DI) was rinsed 3 times with 3 ml sterile saline; ** - the DI was first exposed to *L. mesenteroides* (22.5 min) rinsed (three times with 3 ml sterile saline) and thereafter exposed to *Staph. aureus* spp. *aureus* for 22.5 min; *** - the DI was first exposed to *Staph. aureus* spp. *aureus* (22.5 min), rinsed (three times with 3 ml sterile saline) and thereafter exposed to *L. mesenteroides* for 22.5 min.

Table 2. Light microscopy evaluation of morphological changes of DI of beluga exposed to various bacteria (see Table 1). The results are from 30 micrographs from three fish in treatment 1 to 5 and from 10 micrographs from one fish in treatment 6. Tissue changes were assessed as follows; 0= not observed, 1 = low frequency, 2 = moderate frequency and 3 = high frequency as described by Ringø et al. (2007 b).

Morphological changes	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Disorganised microvillus	0	1	0	1	0	1
Disintegrated tight junctions	0	1	0	1	0	0
Un-normal lamina propria	0	0	0	1	0	0
Loosening of enterocytes from basal membrane	0	2	0	2	0	2
Number of goblet cells	2	3	2	3	2	2
Column totals	2	7	2	8	2	5

29 Table 3. Transmission electron microscopy evaluation of morphological changes of DI of
30 beluga exposed to various bacteria (see Table 1). The results are from 30 micrographs
31 from three fish in treatment 1 to 5 and 10 micrographs from one fish in treatment 6.
32 Tissue changes were assessed as follows; 0= not observed, 1 = low frequency, 2 =
33 moderate frequency and 3 = high frequency as described by Ringø et al. (2007 b).
34

Morphological changes	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Budding from the apices of microvillus	0	1	0	2	1	1
Disorganised microvillus		3	0	2	1	2
Loss of microvillus	0	0	0	1	1	0
Empty goblet cells	2	0	0	0	0	0
Filled goblet cells	1	3	1	1	3	3
Disintegrated tight junctions	0	1	0	2	0	1
Presence of rod let cells	0	0	0	0	0	0
Loosening of enterocytes from basal membrane	0	1	0	2	1	1
Intra epithelial Lymphocyte like cells	0	2	1	1	2	2
Oedema	0	2	0	1	0	3
Column totals	3	13	2	12	9	13

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Table 4

Phylogenetic group	Band no.	Accession no.	Closest relative (obtained from BLAST search)	Identity (%)	Isolation (reference)	Relative abundance (RA, %)															
						101	102	103	104	106	107	108	109	110	111	112	113	118	119	120	121
Proteobacteria	A1	GU301183	*Escherichia coli* BL21(DE3) (CP001509)	100	*Escherichia coli* BL21(DE3), complete genome. (Jeong, unpublished data, NCBI)				4.30	0.60	0.80			2.20							
	A2	GU301184	*Plesiomonas shigelloides* (GQ385072)	97	*Channa striatus* gut microflora (Sarah, unpublished data, NCBI)	3.00	13.70	5.70	2.30	1.40	2.30	1.80	3.20	0.80							
	A4	GU301186	Uncultured *Shewanellaceae* bacterium (EU721817)	93	Oil well; conditions: 27 deg C., 1000 m below surface level, pH 7.4-7.7,USA (Pham et al., 2009)	0.70	21.40	3.80	0.50	2.90	9.60										
	A5	GU301187	*Shewanella* sp. (EF523608)	99	Eutrophic River Warnow, Germany (Freese, unpublished data, NCBI)	17.80	1.00	6.90													
	A6	GU301188	*Aeromonas allosaccharophila* (GQ359956)	98	*Pelteobagrus fulvidraco* intestine content Hubei Province, Niushan Lake (Wu, unpublished data, NCBI)	3.80		0.60					3.70								
	A9	GU301191	*Shewanella* sp. (EU916709)	100	*Clupea harengus* (Baltic hearing) digestive tract (Mickeniene, unpublished data, NCBI)	8.30	3.50	10.50	0.10												
	A10, A11, A13, B2	GU301192	Uncultured *Shewanellaceae* bacterium (EU721794)	100	Oil well; conditions: 27 deg C., 1000 m below surface level, pH 7.4-7.7,USA (Pham et al., 2009)	37.50	31.60	32.70	4.50	1.10	3.80	0.20	1.00	0.10	0.20	8.50	12.20				
	A14	GU301196	Uncultured *Shewanella* sp. (EU073807)	99	Coal enrichment culture,Canada (Penner, unpublished data, NCBI)	3.70															
	A15	GU301197	*Shewanella putrefaciens* (FJ161261)	98	Shandong coast, China (Du, unpublished data, NCBI)	9.10															
	A17, A19, B24	GU301199	*Plesiomonas shigelloides* (GQ385072)	100	*C. striatus* gut microflora (Sarah, unpublished data, NCBI)					5.20	1.10	2.90	1.80	5.30							
	A21	GU301203	*Shewanella putrefaciens* (FJ161261)	100	Shandong coast, China (Du, unpublished data, NCBI)	7.90	0.90	3.90	4.00	0.30	0.10	0.40	0.80	0.40							
	A28	GU301210	*Plesiomonas* sp. (FJ405284)	100	Aquaculture eel, South Korea (Kweon, unpublished data, NCBI)					0.80				0.90							
	A30	GU301212	*Plesiomonas* sp. (FJ405284)	99	Aquaculture eel, South Korea (Kweon, unpublished data, NCBI)					1.30											
	A31, B31	GU301213	*Aeromonas* sp. (AF189694)	100	Activated sludge enriched in nonylphenol ethoxylates, Italy (Barberio et al., 2001)				1.10	3.60	2.30	2.60	4.40	3.40				33.60			
	A33, B29, B33	GU301215	*Aeromonas allosaccharophila* (GQ359956)	100	*P. fulvidraco* intestine content Hubei Province, Niushan Lake (Wu, unpublished data, NCBI)								5.00					10.00	1.00		6.70
	A34	GU301216	*Aeromonas* sp. (FM999973)	100	Diseased *Oncorhynchus mykiss*, Spain (Beaz-Hidalgo et al., 2009)	1.60	9.30	4.10					0.60								
	B9	GU301225	*Acinetobacter* sp. (FJ765352)	100	Surface water sample supplemented with arsenate, Italy (Davolos, unpublished data, NCBI)										6.60	5.30	23.50				
	B17	GU301233	*Aeromonas media* (FJ940831)	99	Water of freshwater fish pond, Wuhan,Hubei, China (Li, unpublished data, NCBI)														5.20		
	B1, B3, B28,	GU301217	*Aeromonas media* (FJ940794)	100	Intestine of silver carp in freshwater pond, Wuhan, Hubei, China (Li, unpublished data, NCBI)													2.10	3.20		8.50

Salma et al. (Table 4 and 5)

	B15, B32	GU301231	*Aeromonas* sp. (AB472996)	100	Fresh fish, Japan: Miyazaki (Tateyama, unpublished data, NCBI)									0.50	0.10	0.10	1.70	15.70	1.40	21.60
Firmicutes	**B6, B7, B8, B11**	GU301222	*Lactobacillus plantarum* (GQ423760)	99	*L. plantarum* used in the present study									26.90	82.70	57.80				
	B21	GU301237	*Staphylococcus aureus* (CP000730)	99	*Staph. aureus* used in the present study												4.20	4.50		18.10
Uncultured bacterium	**A3, B4, B5, B13**	GU301185	Uncultured bacterium (GQ468111)	100	CO_2-treated milk (Rasolofo, unpublished data, NCBI)	0.80	9.20	1.20	3.30	4.20	12.20	0.50					18.70	56.70	84.10	32.80
	A8, B12	GU301190	Uncultured bacterium (EU697160)	98	Atlantic salmon hindgut microbiota (Liu, unpublished data, NCBI)				0.80	2.30	7.60	0.50		3.10			1.10	1.40		1.50
	A12	GU301194	Uncultured bacterium (FM201109)	98	Laboratory-scale membrane bioreactors, Belgium (Huang, De Wever, Diels, 2008)					2.70	7.70	0.80		2.40						
	A16, B20	GU301198	Uncultured bacterium (EU777693)	100	*Ursus maritimus* feces, USA: Saint Louis Zoological Park (Ley et al., 2008)				1.50								0.90			
	A18	GU301200	Uncultured bacterium (GQ359972)	99	*P. fulvidraco* intestine content Hubei Province, Niushan Lake (Wu, unpublished data, NCBI)					0.10	0.50	0.60								
	A20	GU301202	Uncultured bacterium (GQ360015)	99	*P. fulvidraco* intestine content Hubei Province, Niushan Lake (Wu, unpublished data, NCBI)					1.60	0.70	2.20	2.70	0.40						
	A22	GU301204	Uncultured bacterium (GQ166848)	100	Brown bear feces, Norway (Wang, unpublished data, NCBI)	2.10	5.00	27.70	25.00	1.60	1.80	3.80	3.00	0.90						
	A24	GU301206	Uncultured bacterium (GQ360019)	99	*P. fulvidraco* intestine content Hubei Province, Niushan Lake,China (Wu, unpublished data, NCBI)					3.90										
	A27, B23, B27	GU301209	Uncultured bacterium (GQ359972)	100	*P. fulvidraco* intestine content Hubei Province, Niushan Lake,China (Wu, unpublished data, NCBI)				3.80	4.10	4.30	4.90	5.40	4.70			2.20			2.30
	A23, A25, A26, A29, B19, B26, B30	GU301205	Uncultured bacterium (GQ360019)	100	*P. fulvidraco* intestine content Hubei Province, Niushan Lake,China (Wu, unpublished data, NCBI)	3.70	4.00	2.70	42.60	44.00	37.80	66.80	50.90	66.20	65.80	3.40	6.40	13.20	1.70	4.40	4.10
	A32, B25	GU301214	Uncultured bacterium (GQ360019)	99	*P. fulvidraco* intestine content Hubei Province, Niushan Lake,China (Wu, unpublished data, NCBI)				5.10	17.20	6.50	12.00	17.50	8.50						
	B14	GU301230	Uncultured bacterium (EU697160)	99	Atlantic salmon hindgut microbiota (Liu, unpublished data, NCBI)														10.10	
	B22	GU301238	Uncultured bacterium (GQ360015)	100	*P. fulvidraco* intestine mucus, Hubei Province, Niushan Lake,China (Wu, unpublished data, NCBI)												12.30	10.60		4.40
Fungi	**A7, B16**	GU301189	*Saccharomyces* sp. (GQ506978)	100	Cheese whey, Canada (Miao, unpublished data, NCBI)		0.40	0.20	1.10	1.10	0.90			0.70						

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Table 5

	101	**102**	**103**	**104**	**106**	**107**	**108**	**109**	**110**	**111**	**112**	**113**	**118**	**119**	**120**	**121**
101	1.00															
102	0.89	1.00														
103	0.92	0.97	1.00													
104	0.67	0.78	0.75	1.00												
106	0.47	0.58	0.56	0.75	1.00											
107	0.56	0.67	0.64	0.83	0.92	1.00										
108	0.58	0.64	0.61	0.75	0.83	0.92	1.00									
109	0.67	0.67	0.69	0.67	0.64	0.72	0.81	1.00								
110	0.50	0.61	0.58	0.78	0.86	0.89	0.86	0.78	1.00							
111	0.61	0.67	0.64	0.56	0.42	0.50	0.58	0.61	0.56	1.00						
112	0.61	0.67	0.64	0.56	0.42	0.50	0.58	0.61	0.56	1.00	1.00					
113	0.61	0.67	0.64	0.56	0.42	0.50	0.58	0.61	0.56	1.00	1.00	1.00				
118	0.44	0.50	0.47	0.61	0.42	0.50	0.58	0.56	0.50	0.67	0.67	0.67	1.00			
119	0.50	0.56	0.53	0.50	0.36	0.44	0.53	0.50	0.44	0.72	0.72	0.72	0.89	1.00		
120	0.64	0.69	0.67	0.58	0.44	0.53	0.61	0.58	0.53	0.86	0.86	0.86	0.75	0.81	1.00	
121	0.50	0.56	0.53	0.56	0.42	0.50	0.58	0.56	0.50	0.72	0.72	0.72	0.94	0.94	0.81	1.00