Manuscript (AQUA-D-11-00865) resubmitted to "Aquaculture"

Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterisation by 16S rRNA gene sequencing, ability to produce enzymes and *in vitro* growth inhibition of four fish pathogens

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Key words; Atlantic salmon, dietary manipulations, gut microbiota

Abstract

The present investigation evaluated the effect of chitin (5 % supplementation) on the adherent aerobic intestinal microbiota of Atlantic salmon (*Salmo salar* L.). One hundred and seventy three isolates were isolated but 34 isolates died prior to positive identification. Sixty four out of 139 autochthonous gut bacteria were further identified by 16S rRNA gene sequencing and further tested for protease, amylase, cellulase, phytase, lipase and chitinase activities. Moreover, the most promising enzyme-producing bacteria and intestinal lactic acid bacteria (LAB) were tested for *in vitro* growth inhibition of four important fish pathogens: *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio* (*Listonella*) *anguillarum*, *Moritella viscosa* and *Carnobacterium maltaromaticum*. Dietary chitin modulates the gut microbiota but not the portion of enzyme – producing gut bacteria. LAB were only isolated from fish fed the chitin supplemented diet and they were able to inhibit *in vitro* growth of 3 of the 4 pathogens. However, the most promising gut bacteria isolated in the present study with respect to enzyme production and *in vitro* growth inhibition showed high similarity to *Bacillus thuringiensis* by 16S rRNA gene sequencing.

Introduction

Bacteria in the gastrointestinal (GI) tract of fish are considered to be transient (allochthonous), whereas others exist as members of the established microbiota associated with the intestinal mucosa (autochthonous) (Ringø and Birkbeck, 1999; Kim et al., 2007; Merrifield et al., 2009 a; 2009 b). Numerous fish studies have been conducted to characterize the microbial diversity of the GI tract using molecular methods to characterize culturable bacteria (e.g. Holben et al. 2002; Huber et al., 2004; Pond et al., 2006; Ringø et al., 2006 a; 2006 b; Merrifield et al., 2009 b) as well as culture-independent (nonculturable bacteria) studies (e.g. Holben et al., 2002; Huber et al., 2004; Pond et al., 2006; Kim et al., 2007; Liu et al., 2008; Zhou et al., 2009). These investigations have widened the knowledge about the intestinal microbiota in fish, being more complex than previously assumed.

Chitin $(C_8H_{13}O_5N)n$ is a mucopolysaccharide polymer of β 1,4-linked N-acetyl-D-glucosamine residues and is estimated as the second most abundant biomass in the world after cellulose, amounting for approximately $10^6 – 10^7$ tons (Park and Kim, 2010). It forms the basis of the main constituent of the outer exoskeleton of insects and crustaceans like shrimp, crabs and lobster (Kumar, 2000). However, to-day small amounts of chitin are utilized as a material for the aquaculture industry. According to a recent review some information is available about the effect of chitin on total viable counts of gut microbiota (for review see Ringø et al., 2011). However, as only one recent study presents information about modulation of the adherent gut microbiota of Atlantic cod (*Gadus morhua* L.) by supplement of 5 % chitin (Zhou et al., 2011), the first objective of the

present study was to evaluate whether dietary chitin modulates the adherent culturable gut microbiota in the proximal - and distal intestine of Atlantic salmon (*Salmo salar* L.). One of the interesting topics on fish gut bacteria is that some of the gut bacteria produce enzymes that may contribute to fish nutrition (Ray et al., 2011). The 2nd aim of the present study was thus to evaluate whether dietary chitin can increase the frequency of enzyme – producing autochthonous bacteria in the GI tract of Atlantic salmon.

The increased interest during the last decade on lactic acid bacteria (LAB) in the GI tract of fish is related to the fact that LAB often produce bacteriocins and other chemical compounds that may inhibit colonisation of pathogenic bacteria in the GI tract (Ringø et al., 2005; Ringø, 2008; Merrifield et al., 2010; Dimitroglou et al., 2011). Finally, we addressed the issue as to whether LAB isolated in the present study, in addition to the twelve most promising enzyme-producing gut bacteria are able to inhibit *in vitro* growth of four pathogenic bacteria, *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio* (*Listonella*) *anguillarum*, *Moritella viscosa* and *Carnobacterium maltaromaticum*.

Materials and methods

Detailed descriptions of the diets are given by Karlsen et al. (2011). After 115 days of feeding, five fish from each treatment, fed without chitin or with 5 % chitin supplementation, were killed by a sharp blow on the head. The fish were not starved prior to destruction. The ventral belly surface of the fish was opened to expose the peritoneal cavity. The spleen, gallbladder, liver and fat deposits surrounding the gastrointestinal (GI) tract were removed as described by Ringø (1993). The proximal intestine, (PI; defined as the region between the distal pyloric caeca and widening of the intestine and the appearance of transverse luminal folds) and distal intestine, (DI; the region from the widening of the intestine and the appearance of transverse luminal folds to anus) of the digestive tract were excised. Adherent (autochthonous, associated with gut wall tissue) bacteria of the two gut sections were isolated as described elsewhere (Ringø, 1993). Briefly, digesta from PI and DI were gently squeezed out. Thereafter, the two intestinal segments were thoroughly rinsed three times with 3 ml sterile 0.9 % saline solution in order to isolate the autochthonous microbiota. The intestinal segments were transferred to sterile plastic bags and homogenized in a Stomacher (Seward Laboratory, London, UK). Homogenates of the intestinal segments were diluted in sterile 0.9 % saline solution and appropriate dilutions were spread on the surface of Tryptic soy agar (Difco) plates with 5 % glucose and 1 % NaCl. Plates were incubated at 12°C and inspected regularly for up to 4 weeks.

In total 173 isolates isolated from the digestive tract were identified using phenotypic and biochemical methods as described by Ringø and Olsen (1999). Sixty four of these isolates were further identified by 16S rRNA gene sequencing as described by Ringø et al. (2006

a). All sequences were analyzed and edited in BIOEDIT and blasted against the sequences available in GenBank. Isolates showing low similarities (less than 94 %) with known sequences were treated as unknown.

Based on the Blast results, the sequences derived from the gut isolates were aligned with selected sequences from GenBank. A phylogenetic tree of the gut microbiota was subsequently constructed by the Bayesian logarithm using the programs Beast (version 1.6.1.) and Figtree (version 1.3.1).

Gut bacteria identified by 16S rRNA gene sequencing were tested for protease, amylase, cellulase, phytase, lipase and chitinase activities. Bacteria were spread on the surface of peptone – gelatine - agar, starch - agar, carboxymethylcellulose (CMC)-agar, phytate-agar, lipid-agar, and chitin–agar plates, respectively. A detailed description of the media compositions is presented elsewhere (Rapp and Backhaus, 1992; Mondal et al., 2008; Roy et al., 2009; Ray et al., 2010). The cultured plates were incubated at 22^{o}C for 14 days and thereafter washed by different solutions for better clearance of halo zones as described elsewhere (Mondal et al., 2008; Roy et al., 2009). This procedure is important to carry out particularly for detection of amylase, cellulase, phytase and protease activities. Qualitative extracellular enzyme activity was assessed based on the measurement of a clear zone (halo) around the colony as follows; 0 (0 – 3 mm halo zone), 1 (low, 4 - 6 mm halo diameter), 2 (moderate, 7 - 9 mm halo diameter) and 3 (high, > 10 mm halo diameter). Maximum score is 18 and minimum 0.

In vitro growth inhibition of four fish pathogens (*Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio* (*Listonella*) *anguillarum*, *Moritella viscosa* and *Carnobacterium maltaromaticum*) by the most promising enzyme-producing gut bacteria and LAB

126 isolated in the present study was tested using microtitre plate assay as described
127 elsewhere (Ringø et al., 2005; Ringø, 2008; Salma et al., 2011). A detailed description of
128 the pathogens used is presented by Ringø (2008).
129

Results

Table 1 shows log total viable counts (TVC) of autochthonous (adherent) bacteria isolated from the proximal intestine (PI) and distal intestine (DI) of Atlantic salmon fed: (1) control diet and (2) diet supplemented 5 % chitin. One hundred and seventy three autochthonous bacterial strains were isolated from the PI and DI of fish fed the two experimental diets and tried identified based on biochemical and physiological properties. However, 34 isolates died prior to positive identification. Sixty four of the 139 isolates were further identified by 16S rRNA gene sequencing. Of these isolates 8 displayed low similarities to known sequences and were treated as unknown. Identification of the gut bacteria is shown in Tables 1 and 2. The predominant adherent bacteria in PI and DI of fish fed the control diet belonged to *Staphylococcus*, *Bacillus* and *Aeromonas*, while *Staphylococcus*, lactobacilli, *Bacillus* and *Acinetobacter* were dominant in the intestine of fish fed the chitin diet.

Pseudomonas sp. CF8 and *Pseudomonas* – like isolates were only isolated from PI and DI of the control fish. *Psychrobacter cryohalolentis* was only isolated from PI of fish fed the control diet, while *Psychrobacter* sp. ikaite and *Psychrobacter pulmonis* were only isolated from DI of the chitin fed fish. Bacterial strains belonging to *Nesterenkonia* were only isolated from DI of chitin fed fish, while strains identified as *Aeromonas* sp. and *Aeromonas* – like were only isolated from DI of the control fish.

Bacillus cereus and *Bacillus thuringiensis* were only isolated from DI of control fish, while *Bacillus licheniformis* and *Bacillus subtilis* were isolated from chitin fed fish.

In the present study, lactic acid bacteria (LAB) belonging to the *Carnobacterium*, *Leuconostoc* and *Lactobacillus* genera were isolated. Carnobacteria were only isolated

153 from PI of fish fed chitin. With respect to lactobacilli, *Lactobacillus sakei* was isolated
154 from both gut segments of the chitin fed fish while *Leuconostoc citreum* was only
155 isolated from the PI. A further dietary effect was also observed with respect to
156 *Macrococcus equipercicus* and *Macrococcus* – like bacteria and *Micrococcus luteus* and
157 *Micrococcus* – like bacteria. Staphylococci were dominant in the GI tract of both dietary
158 groups but the specific species differed (Table 1).
159 Phylotypes of 64 gut isolates were compared in the BLAST program, and the results with
160 their corresponding accession numbers are displayed in Table 2. These results are based
161 on similarity ≥ 94 % and nucleotides numbering > 800.
162 Representatives of the gut isolates identified by 16S rRNA gene sequencing were chosen
163 for phylogenetic analysis to construct a phylogenetic tree (Figure 1). The phylogenetic
164 tree confirms the results obtained the BLAST function. However, isolate 1113 and 1114
165 with high similarities to *B. cereus* and *B. thuringiensis*, respectively, affiliate within
166 genus *Bacillus*, but not to any particular species.
167 The most promising enzyme producing gut bacteria isolated in the present study are
168 shown in Table 3. Among the isolates characterized by 16S rRNA gene sequencing in the
169 control group, the most promising ones belonged to the genus *Bacillus*. These bacteria
170 had different enzymatic activities, but generally a higher score was noticed with respect
171 to cellulase and chitinase while phytase scores were generally low. The most promising
172 enzyme producing bacteria isolated from the chitin treatment belonged to the genera
173 *Acinetobacter* and *Bacillus*. With respect to highest total enzyme score (> 11) 3 out of 4
174 isolates were isolated from DI of the control group, while 2 out of 3 were isolated from PI
175 of the chitin treatment.

Surprisingly the number of bacteria with high chitinase activity (≥ 2) was highest in strains isolated from the control treatment (6 species) and 3 of them displayed maximum chitinase activity. On the other hand, numbers of bacteria with potential of producing protease and phytase were highest in the chitin treatment. *Carnobacterium* sp., *L. sakei* and *Leu. citreum* were isolated and identified from the chitin group, but in contrast to carnobacteria showing protease and cellulase activities (Table 3B) no enzyme activities were observed regarding to *L. sakei* and *Leu. citreum* (results not shown).

In vitro growth inhibition of *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio* (*Listonella*) *anguillarum*, *Moritella viscosa* and *Carnobacterium maltaromaticum* by isolate 1114 isolated from DI of fish fed the control diet measured by optical density (OD_{600}) is shown in Figure 2. Maximum OD_{600} value (approximately 0.70) of the 4 pathogens were obtained approximately after 16 hours. However, growth of the pathogens was inhibited (OD_{600} = 0.32) when incubated with isolate 1114 supernatant. An overview of the *in vitro* growth inhibition of the four pathogenic bacteria by the supernatant of the most promising enzyme – producing gut bacteria and LAB isolated from Atlantic salmon intestine is shown in Table 4.

Discussion

No effect of dietary chitin was observed on total viable counts of aerobic autochthonous bacteria in proximal intestine (PI) and distal intestine (DI) of Atlantic salmon. However, the present study confirmed previous observations that the bacterial community in fish gut is sensitive to dietary changes (e.g. Sugita et al., 1988; Ringø et al., 1995; Ringø and Olsen, 1999; Ringø et al., 2006 a; 2006 b; Bakke-McKellep et al., 2007).

As conventional culture-based techniques used in the present study only present a partial picture of the microbial diversity of the GI tract, we recommend using molecular methods in future studies evaluating the dietary effect of chitin on the gut microbiota. Therefore, we recently carried out an investigation evaluating the effect of dietary chitin on the gut microbiota in Atlantic cod (*Gadus morhua*) by using PCR-Denaturing Gradient Gel Electrophoresis (Zhou et al., 2011). However, one shall bear in mind that characterization and identification of the gut microbiota designated with its functional role, conventional methods should be used in combination with molecular methods like 16S rRNA / 26S rDNA sequence analysis (in case of bacteria and yeasts, respectively) as suggested in some recent studies (Ghosh et al., 2010; Mondal et al., 2010; Ray et al., 2010).

As several culturable bacterial species were retrieved in the present study that have rarely, or never, previously reported as part of the intestinal microbiota in Atlantic salmon, some general information is therefore presented in the following.

Several studies have reported that *Acinetobacter* appear in the GI tract of fish (e.g. Holben et al., 2002; Ringø et al., 2006 b; Hovda et al., 2007; Merrifield et al., 2009 a; 2009 b). In the present study, 2 strains isolated from PI of fish fed chitin showed high similarity to *Acinetobacter* sp. LUH 1469 and *Acinetobacter* sp. clone S6ABac described by Gundi et al. (2009) and Sharma et al. (2008), respectively.

Previous studies reported that *Aeromonas* are frequently dominant among culturable bacteria in the intestine of fish (for reviews see Cahill, 1990; Sakata, 1990; Ringø et al., 1995). In the present study, 3 strains isolated from PI of the control fed fish displayed high similarity to *Aeromonas* sp. previously described by Goritti et al. (unpublished results, National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/).

The genus *Agrococcus* is a member of the family Microbacteriaceae, and to the author's knowledge, no information is available about *Agrococcus baldri* in the GI tract of fish. Our 16S rRNA gene sequencing analysis displayed 4 strains isolated from PI of fish fed the control diet showing high similarity to *A. baldri* previously reported by An and Yokota (unpublished results, NCBI).

Previous studies reported that species belonging to *Pseudomonas* are frequently dominant among culturable bacteria in fish intestine (for reviews see Cahill, 1990; Sakata, 1990; Ringø et al., 1995). In the present study, 2 strains isolated from PI and DI of the control fed fish displayed high similarity to *Pseudomonas* sp. described by Doulgeraki and Nychas (unpublished, NCBI).

Psychrobacter sp. S3172 was isolated from the GI tract of Atlantic salmon fed chitin. Information about *Psychrobacter* sp. S3172 is available in one unpublished study

evaluating marine culturable bacteria in a global survey of antibacterial activity (Gram et al., unpublished data, NCBI). In the study of Stougaard et al. (2002) evaluating the microbial diversity in ikaite tufa columns in Greenland by analyzing the 16S rRNA genes the authors identified one strain as *Psychrobacter* sp. ikaite c11. In the present study, one strain isolated from DI of fish fed chitin showed high similarity to *Psychrobacter* sp. ikaite c11.

B. subtilis isolated from the chitin treatment was most closely related to a species previously described Toledo et al. (unpublished, NCBI) while *B. licheniformis* was related to a species reported by Zhang et al. (unpublished, NCBI). Of the bacilli strains isolated from control treatment, *Bacillus* sp. YM9-149 and *Bacillus* sp. LM24 were mostly close to species isolated by Goto et al. (unpublished, NCBI) and Zhao (unpublished, NCBI), respectively. The *B. cereus* strains identified in the present study was closely related to the species reported by Tan et al. (unpublished, NCBI) and Krishnani et al. (unpublished, NCBI). *B. cereus* is reported to be a universal soil bacterium but is also reported to be opportunistic pathogen for humans (Helgason et al., 2000). However, *B. cereus* has been used as a probiotic in fish (Nakagawa et al., 2007) and as biological agent against *Aeromonas hydrophila* or for bioremediation in respect to reductions of phosphate, nitrate, nitrite, and ammonia levels in fish cultivation (Lalloo et al., 2007; 2008). It is well known that *B. thuringiensis* play an important role in insecticidal toxins (in spore form) (Helgason et al., 2000), but only one study showed a nontoxic and nonirritant effect of *B. thuringiensis* on fish (Meher et al., 2002). To our knowledge, *B. thuringiensis* has been used as a potential probiotic in one fish study (Reneshwary et al., 2011). This study showed a positive effect of dietary administration

of *B. thuringiensis* on the cellular innate immunity response of African catfish (*Clarias gariepinus*) and against *A. hydrophila* in a challenge study. Based on the results presented in our study with respect to enzyme production and *in vitro* growth inhibition of the pathogens, we recommend that this strains merits further investigations.

Carnobacteria have been isolated from the digestive tract of several fish species (Ringø and Gatesoupe, 1998; Ringø, 2004). The present study isolated one strain of *Carnobacterium* (strain 12266/2009) from DI of chitin fed fish showing high similarity to a strain isolated from human blood culture (Hoenigl et al., 2010) and one strain of carnobacteria isolated from PI of chitin fed fish which shared 94 % similarity to *Carnobacterium* sp. EK-153 previously described by Karelova et al. (NCBI, unpublished results).

Allochthonous *Lactobacillus sakei* have been isolated from the digestive tract of several fish species (Gonzalez et al., 2000; Bucio et al., 2006; Balcazar et al., 2007; Ghanbari et al., 2009; Hagi and Hoshino, 2009). However, to our knowledge autochthonous *L. sakei* has not previously been isolated from the GI tract of fish. The 4 autochthonous strains isolated in the present study were most closely related to *L. sakei* isolated from traditional fermented food in Taiwan (Chang and Chen, unpublished results, NCBI).

Some information is available on *Leuconostoc citreum* in the GI tract of fish (Han et al., 2010; Sica et al., 2010). In the present study one isolate was most closely related to *L. citreum* previously isolated from fermented ginger in Taiwan (Chen and Chang, unpublished, NCBI).

To our knowledge *Macrococcus equipercicus* has not previously been isolated from the GI tract of fish, but in the present study one strain isolated from DI of fish fed chitin

283 showed high similarity to *M. equipercicus* isolated from the skin of Irish thoroughbred
284 horse, Morgan horse and Shetland ponies (Kloos et al., 1998).
285 Two stains isolated from the PI of fish fed the control diet showed high similarity to
286 *Micrococcus luteus* previously described by Edward et al. (unpublished results, NCBI).
287 A phylogenetic and chemotaxonomic re-analysis of the genus *Micrococcus* resulted in the
288 proposal of the genus *Nesterenkonia* (Stackebrands et al., 1995). Information on
289 *Nesterenkonia* sp. YIM70084 has been presented by Li et al. (2004) in a study isolating
290 actinobacteria from saline soils in China. In our study, two autochthonous strains were
291 isolated from DI of fish fed chitin that showed high similarity *Nesterenkonia* sp.
292 YIM70084.
293 During the last decade, some studies have presented information on the presence of
294 *Staphylococcus* in the GI tract of fish (Esteve and Garay, 1991; Ringø et al., 2006 a; 2006
295 b; Bakke-Mc Kellep et al., 2007). In the present study we identified 24 adherent strains
296 most closely related to *Staphylococcus equorum*, *Staphylococcus pasteuri*,
297 *Staphylococcus warneri* and *Staphylococcus* sp. WPCB124,
298 The present study showed that supplementation of 5 % chitin did not affect the
299 population level of culturable adherent bacteria, but the supplementation modulated the
300 adherent gut microbiota. In this respect a fundamental question arises: does the GI tract
301 microbiota have a protective role against pathogenic colonisation? During the last 25
302 years, numerous papers have suggested that the alimentary tract is involved in *Aeromonas*
303 and *Vibrio* infections (for reviews see Ringø et al., 2003; Birkbeck and Ringø, 2005;
304 Ringø et al., 2007), therefore, one can hypothesise that beneficial bacteria colonising the
305 GI tract may offer protection against invading fish pathogens. *In vitro* growth inhibition

306 of *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio* (*Listonella*) *anguillarum*,
307 *Moritella viscosa* and *Carnobacterium maltaromaticum* showed that *B. thuringiensis* and
308 the LAB isolated in the present study have antagonistic activities. However, in order to
309 clarify whether supplementation of chitin improve disease resistance, challenge studies
310 have to be carried out.
311 Numerous studies have focused on the functional relationship between the beneficial gut
312 microbiota, enzyme producing bacteria, and their contribution to fish nutrition (Ray et al.,
313 2011). The presence of LAB was detected in the gut of fish fed 5% chitin treatment.
314 However, no or relatively low extracellular enzymatic activities were observed in this
315 bacterial group, but they had good *in vitro* growth inhibition against 3 of the 4 pathogens
316 tested. Whether the positive effect of chitin inclusion on gut LAB has any protective
317 effect merits further evaluations.
318
319 ***Conclusions and further perspectives***.
320 The present study clearly displayed that dietary chitin modulated the gut microbiota of
321 Atlantic salmon. Furthermore, the proportion of enzyme – producing gut bacteria and the
322 ability of gut bacteria to inhibit growth of four well known pathogenic bacteria was
323 affected by dietary manipulation. Whether the beneficial bacteria isolated in the present
324 study have any effect on fish growth and disease resistance merits further investigations.
325 One of the most promising enzyme – producing bacteria isolated, *B. thuringiensis*, also
326 displayed promising antibacterial activities against all the fish pathogenic bacteria tested.
327 Whether this strains is applicable as a probiotics in Atlantic salmon rearing merits further
328 investigations.

Acknowledgements

This project was a co-operation project between Norway and China. Financial support from Norwegian Research Council (project no. 199812-S40) is gratefully acknowledged. The authors kindly thank Dr. Daniel L. Merrifield, University of Plymouth, UK for his constructive comments on the manuscript.

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519

520

521 Table 1. Log total viable counts (TVC) of autochthonous bacteria isolated from the
522 proximal intestine (PI) and distal intestine (DI) of Atlantic salmon (*Salmo salar* L.) fed
523 control diet (1) and diet supplemented with 5 % chitin (2).

	Diet 1	Diet 1	Diet 2	Diet 2
	PI	**DI**	**PI**	**DI**
Log TVC	5.45	5.91	5.93	5.64
Gram-negatives				
*Acinetobacter johnsonii**	3.79	4.27		
Acinetobacter sp.*			4.61	
Acinetobacter – like	4.10	4.27	4.61	
Aeromonas sp.*		4.74		
Aeromonas – like		4.74		
*Agrococcus baldri**	4.39			
Agrococcus – like	4.10			
Pseudomonas sp.*	3.79	4.27		
Pseudomonas – like	4.10	4.56		
*Psychrobacter cryohalolentis**	3.79			
Psychrobacter sp. ikaite*				4.02
*Psychrobacter pulmonis**				4.02
Gram – negative rods**	4.27	4.74	4.91	4.02
Gram-positives				
*Bacillus cereus**		4.56		
*Bacillus licheniformis**			4.31	4.02
*Bacillus subtilis**				4.32
Bacillus thuringiensis		4.27		
Bacillus sp.*	4.10			
Bacillus – like	4.49	4.96	4.61	4.50
Carnobacterium sp. 12266/2009*			4.61	
Carnobacterium sp. EK-153*			4.31	
*Lactobacillus sakei**			4.61	4.32
Lactobacillus - like			4.91	4.72
*Leuconostoc citreum**			4.31	
*Macrococcus equipercicus**				4.02
Macrococcus - like				4.50
*Micrococcus luteus**	4.10			
Micrococcus - like	4.10			
Nesterenkonia sp. YIM70084*				4.02
Nesterenkonia – like				4.62
*Staphylococcus equorum**			5.16	4.72

*Staphylococcus pasteuri**	4.27	4.56		
*Staphylococcus warneri**	3.79	4.27		
Staphylococcus sp.*	3.79	4.74		4.02
Staphylococcus – like	4.49	5.04	5.01	4.62
Uncultured bacterium clone ncd2745e02c1*		4.74		
Gram – positive rods**	4.39	4.74	4.31	4.02
Gram – positive cocci**	4.39	4.87	4.91	4.32
Unknown***			4.91	4.62

524
525 * – identified by 16S rRNA; **- isolates died prior to positive identification;
526 *** - isolates showing poor sequences are treated as unknown.
527
528

529 Table 2. Identification of bacterial strains isolated from intestine of Atlantic salmon fed
530 control diet (diet 1) and diet supplemented with 5 % chitin (diet 2) with partial sequence of
531 16S rRNA genes referenced to accession no in GenBank.

Strain no.	Closest relative (obtained from BLAST search)	Accession no	Similarity (%)	No of strains showing high similarity to the closest relative	Isolated from
978	*Acinetobacter johnsonii*	HQ739094	99	2	PIDI-1
987	*Acinetobacter* sp. clone S6ABac	EU669181	98	1	PI-2
988	*Acinetobacter* sp. LUH1469	FJ860877	98	1	PI-2
1119	*Aeromonas* sp.	FR799758	98	3	PIDI-1
979	*Agrococcus baldri*	AB29548	99	4	PI-1
966	*Pseudomonas* sp.	HQ014882	99	2	PIDI-1
961	*Psychrobacter cryohalolentis*	EU090718	98	1	PI-1
1012	*Psychrobacter pulmonis*	EF101551	98	1	DI-2
956	*Psychrobacter* sp. ikaite c11	AJ431338	98	1	DI-2
1113	*Bacillus cereus*	JF264468	99	2	DI-1
1007	*Bacillus licheniformis* strain Y822	HQ005269	96	2	PIDI-2
1015	*Bacillus subtilis* strain DmB4	HQ111352	99	2	DI-2
1114	*Bacillus thuringiensis*	EU874887	100	1	DI-1
1112	*Bacillus* sp.	AB243862	94	2	PI-1
983	*Carnobacterium* sp. 12266/2009	GQ281028	98	2	PI-2
1022	*Carnobacterium* sp. EK-153	GU935293	94	1	PI-2
997	*Lactobacillus sakei* 1101	AB593361	99	4	PIDI-2
980	*Leuconostoc citreum* 4501	AB593366	98	1	PI-2
996	*Macrococcus equipercicus*	Y15712	96	1	DI-2
976	*Micrococcus luteus*	HM449702	99	2	PI-1
1010	*Nesterenkonia* sp. YIM70084	AY226508	99	1	DI-2
1016	*Staphylococcus equorum*	AB334773	99	12	PIDI-2
975	*Staphylococcus pasteuri*	FJ435675	97	5	PIDI-1
1102	*Staphylococcus warneri*	HQ284960	100	2	PIDI-1
971	*Staphylococcus* sp.	HQ677396	100	2	DI-1
1001	*Staphylococcus* sp. 09BS3-3	HM565997	98	3	DI-2
963	Uncultured bacterium clone ncd2745e02c1	JF236822	99	3	DI-1
	Total no. of strains identified by 16S rRNA gene sequencing			64	

532
533 PI-1 isolated from proximal intestine of fish fed diet 1; DI-1 isolated from distal intestine of
534 fish fed diet 1; PIDI-1 isolated from both proximal – and distal intestine of fish fed diet 1; PI-
535 2 isolated from proximal intestine of fish fed diet 2; DI-2 isolated from distal intestine of fish
536 fed diet 2; PIDI-2 isolated from both proximal – and distal intestine of fish fed diet 2.
537

Table 3. Enzyme – producing bacteria, the most promising ones. A - isolated from the GI-tract of Atlantic salmon fed control diet and B – isolated from the GI-tract of Atlantic salmon fed 5% chitin. Number of tested bacteria from the control group = 51. Number of tested bacteria from the chitin group =36.

A

Strain no.	Protease (score)	Amylase (score)	Cellulase (score)	Phytase (score)	Lipase (score)	Chitinase (score)	Total score	Closest relative (obtained from BLAST search)	Accession no.
966**	1	2	2	0	2	0	7	*Pseudomonas* sp.	HQ014882.1
978*	0	3	2	1	0	2	8	*Acinetobacter johnsonii*	HQ739094.1
979*	0	3	2	1	0	2	8	*Agrococcus baldri*	AB279548.1
1114**	2	0	3	1	3	3	12	*Bacillus thuringiensis*	EU874887.1
1113**	2	0	3	1	3	3	12	*Bacillus cereus*	JF264468.1
1112*	2	3	3	1	2	2	13	*Bacillus* sp.	HQ891939.1
1115**	3	0	3	1	3	3	13	*Bacillus cereus*	HQ833025.1

B

Strain no.	Protease (score)	Amylase (score)	Cellulase (score)	Phytase (score)	Lipase (score)	Chitinase (score)	Total score	Closest relative (obtained from BLAST search)	Accession no.
983*	3	0	3	0	0	0	6	*Carnobacterium* sp.	GQ281028.1
1022*	3	0	3	0	0	0	6	*Carnobacterium* sp.	GU935293.1
995*	3	0	3	0	0	0	6	*Staphylococcus equorum*	HQ202869.1
1001**	2	3	2	1	0	0	8	*Staphylococcus* sp.	HM565997.1
1015**	2	0	2	1	3	3	11	*Bacillus subtilis*	HQ111352.1
987*	3	2	2	2	1	2	12	*Acinetobacter* sp.	EU669181.1
988*	3	1	2	3	1	2	12	*Acinetobacter* sp.	FJ860877.1

* - isolated from proximal intestine; ** - isolated from distal intestine

Table 4. *In vitro* growth inhibition of *A. salmonicida*, *V. anguillarum*, *M. viscosa* and *C. maltaromaticum* by the most promising enzyme – producing bacteria and lactic acid bacteria isolated from the digestive tract of Atlantic salmon fed control diet (A) and 5 % chitin (B). PI – proximal intestine; DI – distal intestine; + growth inhibition; - growth inhibition.

A

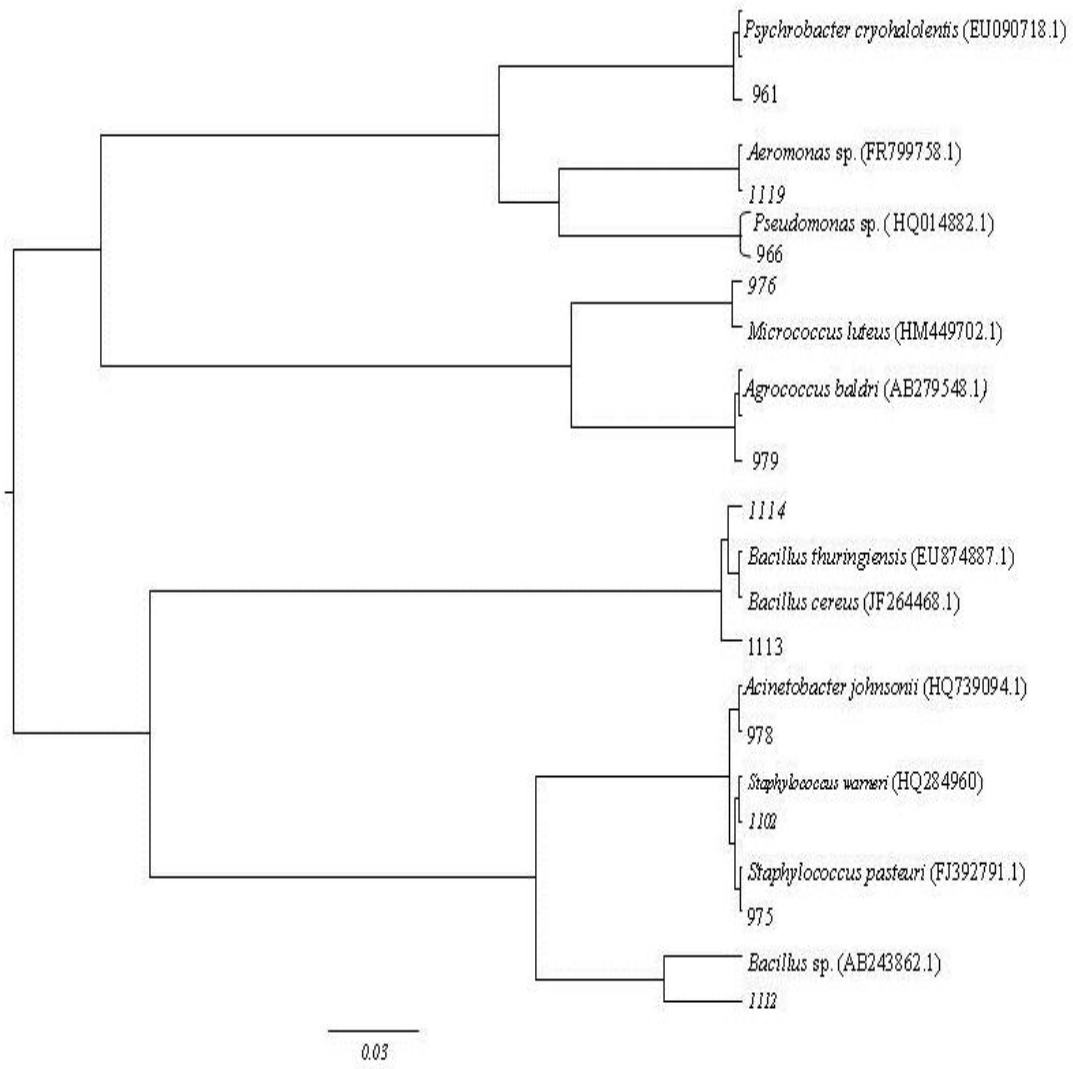
Gut isolate showing high similarity to	**Accession No.**	**Isolated from**	**Growth inhibition of** *A. salmonicida*	**Growth inhibition of** *V. anguillarum*	**Growth inhibition of** *M. viscosa*	**Growth inhibition of** *C. maltaromaticum*
Pseudomonas sp.	HQ014882.1	DI	-	-	-	-
Acinetobacter johnsonii	HQ739094.1	PI	-	+	+	-
Agrococcus baldri	AB279548.1	PI	+	+	-	+
Bacillus thuringiensis	EU874887.1	DI	+	+	+	+
Bacillus cereus	JF264468.1	DI	+	+	-	-
Bacillus sp.	HQ891939.1	PI	-	-	-	-
Bacillus cereus	HQ833025.1	DI	+	-	-	-

B

Gut isolate showing high similarity to	**Accession No.**	**Isolated from**	**Growth inhibition of** *A. salmonicida*	**Growth inhibition of** *V. anguillarum*	**Growth inhibition of** *M. viscosa*	**Growth inhibition of** *C. maltaromaticum*
Carnobacterium sp.	GQ281028.1	PI	+	+	+	-
Carnobacterium sp.	GU935293.1	PI	+	+	+	-
Staphylococcus equorum	HQ202869.1	PI	-	-	-	-
Staphylococcus sp.	HM565997.1	DI	-	-	-	-
Bacillus subtilis	HQ111352.1	DI	+	-	-	-
Acinetobacter sp.	EU669181.1	PI	-	-	-	-
Acinetobacter sp.	FJ860877.1	PI	-	-	-	-
Lactobacillus sakei	AB593361.1	DI	+	+	+	-
Lactobacillus sakei	GQ449257.1	PI	+	+	+	-
Lactobacillus sakei	EU135690.1	PI	+	+	+	-
Leuconostoc citreum	AB593366.1	PI	-	+	+	-

Figure 1. Phylogenetic tree of the gut microbiota isolated from; (A) fish fed control diet and (B) fish fed 5% chitin. Sequences were aligned with BioEdit and a Bayesian phylogenetic reconstruction was performed. The accession numbers of GenBank sequences are given in brackets.

A



B

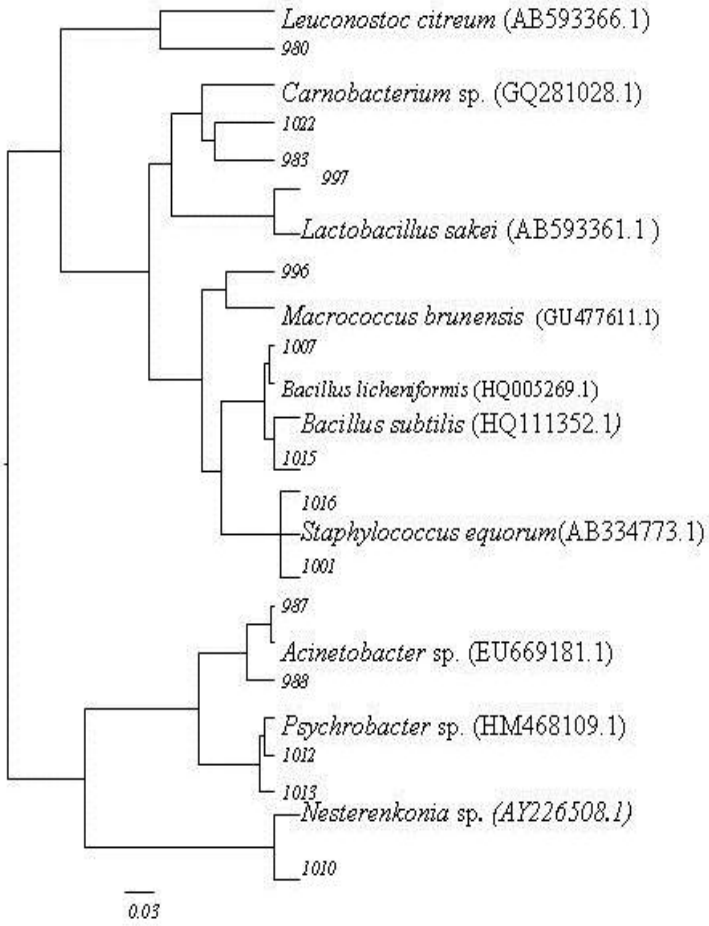

Leuconostoc citreum (AB593366.1)
980
Carnobacterium sp. (GQ281028.1)
1002
983
997
Lactobacillus sakei (AB593361.1)
996
Macrococcus brunensis (GU477611.1)
1007
Bacillus licheniformis (HQ005269.1)
Bacillus subtilis (HQ111352.1)
1015
1016
Staphylococcus equorum(AB334773.1)
1001
987
Acinetobacter sp. (EU669181.1)
988
Psychrobacter sp. (HM468109.1)
1012
1013
Nesterenkonia sp. (AY226508.1)
1010
0.03

Figure 2. *In vitro* growth inhibition of (A) *C. maltaromaticum*, (B) *M. viscosa*, (C) *V. anguillarum* and (D) *A. salmonicida* by supernatant of isolate 1114 isolated from DI of fish fed the control diet. Black line – control (absence of isolate 1114 supernatant). Blue line –culture of pathogen with isolate 1114 supernatant.