Short communication (ARE-SC-11-Jul-522.R2) resubmitted to "Aquaculture Research"

Accepted for publication 2011-11-09

The effect of different feeding regimes on enzyme activities of gut microbiota in Atlantic cod (*Gadus morhua* L.)

Fatemeh Askarian[1,*], Sigmund Sperstad[1], Daniel L. Merrifield[2], Arun Kumar Ray[3] and Einar Ringø[1,4]

[1]Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Norway.

[2]Aquaculture and Fish Nutrition Research Group, School of Biomedical and Biological Sciences, The University of Plymouth, UK.

[3]Fisheries Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan, West Bengal, India.

[4] Aquaculture Protein Centre (a CoE), Department of Aquatic Medicine and Nutrition, Norwegian School of Veterinary Medicine, P.O. Box 8146 Dep., No-0033 Oslo, Norway.

* Corresponding author: E-mail: fatemehaskarian@yahoo.com

Key words; Atlantic cod, feeding regimes, gut microbiota, enzymatic activities, *in vitro* growth inhibition of pathogens

The presence of autochthonous gut microbiota in fish has been reported in numerous studies (e.g. Cahill 1990; Ringø, Strøm & Tabachek 1995; Birkbeck & Ringø 1999; Austin 2006; Merrifield, Dimitroglou, Foey, Davies, Baker, Bøgwald, Castex & Ringø 2010; Nayak 2010; Merrifield, Olsen, Myklebust & Ringø 2011). With respect to autochthonous gut microbiota in Atlantic cod (*Gadus morhua* L.) some information is available. Seppola, Olsen, Sandaker, Kanapthippillai, Holzapfel & Ringø (2006) presented information on carnobacteria in the hindgut and hindgut chamber, while Ringø, Sperstad, Myklebust, Refstie & Krogdahl (2006 a) investigated the effect of different feeding regimes on the gut microbiota of Atlantic cod. Later, Lauzon, Gudmundsdottir, Petursdottir, Reynisson, Steinarsson, Oddgeirsson, Bjornsdottir & Gudmundsdottir (2007) isolated probiotic bacteria from cod rearing environment and the gastrointestinal (GI) tract of cod juveniles. Løvmo Martinsen, Salma, Myklebust, Mayhew & Ringø (2011) addressed whether the midgut of Atlantic cod is a site of colonization for *Vibrio (Listonella) anguillarum* and if *Carnobacterium*, a probiotic bacterium, is able to out-compete the pathogen and modulate the adherent gut microbiota. However, to the author`s knowledge, there is no information available regarding enzyme-producing bacteria isolated from Atlantic cod intestine. This topic is relevant to evaluate as some reviews have suggested that gut microbiota can contribute to fish digestive function (Ringø *et al*. 1995; Austin 2006; Nayak 2010; Ray, Gosh & Ringø 2011).

The gut microbiome is important in fish health (Gómez & Balcázar 2007; Nayak 2010; Merrifield *et al*. 2010) and it has been suggested that the autochthonous gut microbiota could inhibit colonization of pathogenic bacteria by mechanisms including space occupation, competition of nutrients, blocking receptors on mucosal surface and production of antagonistic compounds (e.g. Gatesoupe 1999; Ringø, Schillinger & Holzapfel 2005; Ringø *et*

al. 2006 a; Caipang, Brinchmann & Kiron 2010). However, to our knowledge, antagonistic activity of gut bacteria isolated from the GI tract of Atlantic cod has only been investigated in two studies (Ringø *et al*. 2006 a; Caipang *et al*. 2010).

The aims of the present study were: (1) evaluate enzyme-producing bacteria isolated from the GI tract of Atlantic cod, (2) identify the most promising enzyme-producing bacteria by 16S rRNA gene sequencing and (3) to assess whether these bacteria have the ability to inhibit *in vitro* growth of four well known pathogenic bacteria; *Aeromonas salmonicida* subsp. *salmonicida*, *V*. (*L*.) *anguillarum*, *Moritella viscosa* and *Carnobacterium maltaromaticum.*

In the present study, 79 gut bacteria previously isolated from the GI tract of Atlantic cod fed fish meal (FM), soybean meal (SBM) and bioprocessed soybean meal (BPSBM) from the study of Ringø *et al*. (2006 a), were randomly selected for further investigation. These bacteria had not previously been tested for enzyme-production, identified by 16S rRNA gene sequencing or tested for antagonistic activity. Determination of qualitative enzyme activities; protease, amylase, cellulase, phytase, lipase and chitinase were carried out as described by Ray, Roy, Mondal & Ringø (2010) and Askarian, Zhou, Olsen, Sperstad & Ringø (2011). These endogenous bacterial enzymes were selected as they might contribute to fish nutrition (Ray *et al*. 2011). Forty eight of the most promising enzyme-producing bacteria, 15, 16 and 17 isolated from the GI tract of Atlantic cod fed FM, SBM and BPSBM, respectively were further identified by 16S rRNA gene sequencing as described by Ringø, Sperstad, Myklebust, Mayhew & Olsen (2006 b). All sequences were analyzed and edited in BIOEDIT and blasted against the sequences available in GenBank. Gut bacteria showing low similarities (< 94 %) with known sequences in GenBank were treated as unknown.

In vitro growth inhibition of four fish pathogens (*A. salmonicida* subsp. *salmonicida*, *V*. (*L*.) *anguillarum*, *M. viscosa* and *C. maltaromaticum*) by the most promising enzyme-producing gut bacteria was tested using a microtitre plate assay (Ringø *et al*. 2005; Ringø 2008; Salma, Zhou, Wang, Askarian, Kousha, Ebrahimi, Myklebust & Ringø 2011; Askarian *et al*. 2011). Bacterial growth was estimated at optical density (OD_{600} nm) for 48 hours at 30°C. An automatic plate reader (Bioscreen C. Lab systems, Finland) was used to measure bacterial growth (each hour) and inhibition of growth was defined when OD_{600} was reduced by 50% or more. A detailed description of the pathogens used in the present study is given by Ringø (2008).

The most promising enzyme-producing bacteria isolated from the GI tract of Atlantic cod are presented in Table 1, and the diversity seems to be influenced by the feeding regimes. The most promising enzyme-producing bacteria isolated from FM fed fish was similar to *Brochothrix* sp. (accession no. HQ890945.1) and had a score of 10 out of 18 (10/18). This isolate exhibited high (score 3) protease and cellulase activities but moderate chitinase and amylase activities (Table 1A). This bacterium was isolated from both the fore -, mid - and hindgut of Atlantic cod. Furthermore, 3 other isolates showing high similarity to *Psychrobacter cryohalolentis*, *Brochothrix thermosphacta* and *Psychrobacter* sp., displayed high protease activity (Table 1A). Moreover, *Brochothrix* sp. and *P. cryohalolentis* were the only strains, of all the isolates tested, which displayed high cellulase activity (Table 1). The most promising enzyme-producing gut bacteria isolated from SBM group, with a score of 9/18, was similar to *Brochothrix* sp. (accession no. AM409367.1) and was isolated from the foregut. This bacterium displayed high lipase and chitinase activities, moderate levels of protease and cellulase activities, but low levels of phytase and amylase activities (Table 1B). The *Brochothrix* sp. isolated from the SBM treatment was the only isolate out of all isolates investigated with high lipase activity (Table 1). *Brochothrix* sp., *Psychrobacter* sp.,

Carnobacterium sp. and *Staphylococcus equorum* displayed high protease and to some extent phytase activities. Surprisingly, no amylase activity was detected in the most promising enzyme-producing bacteria isolated from the SBM treatment.

Brochothrix thermosphacta, with a score of 7/18, was identified as the most promising enzyme-producing bacteria in BPSBM treatment with maximum protease, moderate lipase and low phytase activities (Table 1 C). Generally, the most promising enzyme-producing bacteria isolated from BPSBM treatment, showed low or no cellulase activity. The ability for extracellular secretion of protease varied from being completely absent (*Jeotgalibacillus* sp.) to high (*Psychrobacter* sp. and *B. thermosphacta*). Two isolates displaying high similarity to the *Jeotgalibacillus* and *Pseudomonas* genera, showed maximum amylase activity; these strains, of all the isolates tested, were the only isolates which displayed high amylase activity (Table 1). However, the most promising enzyme-producing bacteria isolated from the BPSBM treatment showed lower total enzymatic activities compared to bacteria tested from the other treatments.

The results of the *in vitro* growth inhibition assays are displayed in Table 2. Of the 9 isolates tested, only, *Carnobacterium* sp. was able to inhibit all four pathogens. However, the most promising-enzyme producing bacteria (*Brochothrix* sp.) isolated from FM and SBM displayed inhibitory *in vitro* effect against *A. salmonicida*, *V.* (*L.*) *anguillarum* and *M. viscosa*. In contrast, *Brochothrix thermosphacta*, the most promising enzyme-producing bacteria isolated from BPSBM treatment showed no inhibitory effect against the pathogens tested.

As described by Ringø & Birkbeck (1999), the gut microbiota can be divided into autochthonous (indigenous) and allochthonous (transient) bacteria. In the present study, autochthonous bacteria were tested for enzymatic activities. These isolates were previously isolated from gut of Atlantic cod by Ringø *et al*. (2006 a). The present study identified some enzyme-producing bacteria that have rarely been reported in the fish gut. Gut bacteria belonging to *Brochothrix* sp., *B. thermosphacta* and *Jeotgalibacillus* sp. were among the most promising enzyme-producing bacteria in the gut of Atlantic cod.

Psychrobacter sp. was identified as one the most active digestive enzyme-producing bacterium in all treatments. This bacterium showed high similarity to *Psychrobacter* sp. clone B5-2 previously reported Li, He & Matthias (unpublished data, National Center for Biotechnology Information (NCBI)) from enrichment culture. According to the authors` knowledge enzymatic activities of *Psychrobacter* sp. has not been reported previously. *Psychrobacter cryohalolentis* isolated from midgut of Atlantic cod fed with FM showed high similarity to *P. cryohalolentis* strain KOPRI_22219 reported by Lee, Jung, Cho, Cho, Hong & Yim (unpublished data, NCBI), and had a total score of 8/18 with respect to enzymatic activities. It displayed high protease and cellulase activities, moderate amylase activities and was able to inhibit *in vitro* growth of *A. salmonicida*. To our knowledge, the present study is the first report of antagonistic activity of *P. cryohalolentis* against *A. salmonicida*.

In the present study, we isolated two strains belonging to genus *Brochothrix* from the FM and SBM treatments and these strains displayed high similarity to *Brochothrix* sp. MVP25 and *Brochothrix* sp. NJ-25 previously reported by Nowak, Oltuszak-Walczak & Walczak (unpublished data, NCBI) and Gai (unpublished data, NCBI), respectively. These strains had inhibitory effect against 3 of the pathogens tested, except for *C. maltaromaticum*. Isolate 511, from the FM treatment, showed high similarity to *B. thermosphacta* strain ATCC 11509 reported by Nowak, Oltuszak-Walczak & Walczak (unpublished data, NCBI). To our knowledge, the enzyme activities of *Brochothrix* sp. and *B. thermosphacta* are presented for

the first time in the present study. To the authors` knowledge, there are no reports available about pathogenicity of *B. thermosphacta*.

During the last decade, numerous studies have demonstrated antagonistic activities of beneficial gut bacteria against fish pathogens (e.g. Irianto & Austin 2002; Balcázar, de Blaz, Ruiz Zarzuela, Cunningham, Vendrell & Múzquiz 2006; Ringø *et al*. 2005; 2006 a; Ringø 2008; Askarian *et al*. 2011; Pérez-Sánchez, Balcázar, García, Halaihel, Vendrell, Blas, Merrifield & Ruiz-Zarzuela 2011; Salma *et al*. 2011). Furthermore, different mechanisms such as lower pH, elevated immune responses, production of antibacterial substances, competition for nutrients and colonization in the GI tract have been proposed for antagonistic action of beneficial bacterial against well-known fish pathogens *in vivo* (e.g. Ringø and Birkbeck 1999; Irianto & Austin 2002; Ringø *et al*. 2005; Merrifield *et al*. 2010; Nayak 2010; Pérez-Sánchez *et al*. 2011).

Carnobacterium sp. strain 476 isolated from the SBM treatment was closely related to *Carnobacterium* sp. I-Bh4-26 previously reported by Baker, Schwarz & Conrad (2010). An interesting finding of the present study was that *Carnobacterium* sp. strain 476 displayed antagonistic effect against all the tested pathogens.

According to Table 1, promising enzyme-producing bacteria were isolated from all gut sections of Atlantic cod. However, 11 out of 13 strains of the most promising enzyme-producing bacteria in the different treatments were isolated from the foregut of Atlantic cod. Based on these results, we put forward the hypothesis that the foregut is the main part of gut for isolation of enzyme-producing bacteria in Atlantic cod. However, to confirm this hypothesis further studies have to be carried out.

The present study demonstrated that different feeding regimes; FM, SBM and BPSBM influence diversity and endogenous enzyme activities of the most promising enzyme-producing bacteria in Atlantic cod intestine. For example, maximum protease activity was detected in all of the promising strains isolated from the FM treatment while no amylase activity was noticed among the strains isolated from the SBM treatment. Furthermore, cellulase activity was only detected in one out of the 5 most promising strains isolated from the BSBM treatment. This finding may be a dietary effect, but further investigations are needed. Whether the beneficial bacteria reported in the present study has any effects as growth promoters or improves disease resistance of Atlantic cod merits further investigations.

Acknowledgment

The authors thank Hans-Matti Blencke, University of Tromsø, for his help during the *in vitro* growth inhibition tests.

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Table 1. Enzyme – producing bacteria, the most promising ones isolated from the gut of Atlantic cod fed; fish meal (A), soybean meal (B) and bioprocessed soybean meal (C). Number of tested gut bacteria isolated from the fish meal, soybean meal and bioprocessed soybean meal group were 25, 26 and 28, respectively.

A

Strain no.	Protease (score)	Amylase (score)	Cellulase (score)	Phytase (score)	Lipase (score)	Chitinase (score)	Total score	Organisms with closest 16S rRNA gene sequence in GenBank	Accession no.
511	3[d]	0[a]	0	1[b]	2[c]	1	7	*Brochothrix thermosphacta*[****]	HQ890942.1
505	3	2	3	0	0	0	8	*Psychrobacter cryohalolentis*[**]	EU090718.1
506	3	2	3	0	0	2	10	*Brochothrix* sp.[****]	HQ890945.1
518	3	0	0	2	0	1	6	*Psychrobacter* sp.[*&**]	GU570650.1

B

Strain no.	Protease (score)	Amylase (score)	Cellulase (score)	Phytase (score)	Lipase (score)	Chitinase (score)	Total score	Organisms with closest 16S rRNA gene sequence in GenBank	Accession no.
478	3	0	0	2	0	1	6	*Psychrobacter* sp.[*&***]	GU570650.1
491	3	0	2	1	0	0	6	*Staphylococcus equorum*[****]	HM163522.1
476	2	0	2	1	0	0	5	*Carnobacterium* sp.[***]	FN555396.1
485	2	0	2	1	3	3	9	*Brochothrix* sp.[*]	AM409367.1

C

Strain no.	Protease (score)	Amylase (score)	Cellulase (score)	Phytase (score)	Lipase (score)	Chitinase (score)	Total score	Organisms with closest 16S rRNA gene sequence in GenBank	Accession no.
520	3	0	0	2	0	1	6	*Psychrobacter* sp.[****]	GU570650.1
522	3	0	0	1	2	1	7	*Brochothrix thermosphacta*[*&***]	HQ890942.1
523	2	0	1	2	0	1	6	Uncultured bacterium[****]	JF011078.1
525	1	3	0	0	1	0	5	*Pseudomonas* sp.[*]	HQ014889.1
528	0	3	0	0	0	1	4	*Jeotgalibacillus* sp.[*]	DQ069205.1

*- foregut; ** - midgut; *** - hindgut; **** - all 3 segment of the intestine

Ranking of halo zone around the colony; [a] - 0 (< 4 mm), [b] - 1 (low, 4 - 6 mm), [c] - 2 (moderate, 7 - 9 mm) and [d] - 3 (high, > 10 mm). Maximum score is 18 and minimum 0.

255 Table 2. *In vitro* growth inhibition* of *A. salmonicida*, *V. anguillarum*, *M. viscosa* and *C.*
256 *maltaromaticum* by the most promising enzyme – producing bacteria isolated from the digestive
257 tract of Atlantic cod fed; fish meal (A), soybean meal (B) and bioprocessed soybean meal (C).

Closest relative (obtained from BLAST search)	**Accession No.**	**Isolated from the GI tract of fish fed diet**	**Growth inhibition of** *A. salmonicida*	**Growth inhibition of** *V. anguillarum*	**Growth inhibition of** *M. viscosa*	**Growth inhibition of** *C. maltaromaticum*
Psychrobacter cryohalolentis	EU090718.1	A	+	-	-	-
Brochothrix sp.	AM409367.1	B	+	+	+	-
Brochothrix sp.	HQ890945.1	A	+	+	+	-
Brochothrix thermosphacta	HQ890942.1	A & C	-	-	-	-
Psychrobacter sp.	GU570650.1	B & C	-	-	-	-
Uncultured bacterium	JF011078.1	C	-	-	-	-
Pseudomonas sp.	HQ014882.1	C	-	-	-	-
Jeotgalibacillus sp.	DQ069205.1	C	-	-	-	-
Carnobacterium sp.	FN555396.1	B	+	+	+	+

258 *; + ≥ 50 % growth inhibition; - < 50 % growth inhibition.

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260