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Review

The causes of stress on intestinal microbiota of Atlantic salmon, Arctic charr, Atlantic cod and rainbow trout

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The gastrointestinal (GI) tract of fish reacts to stress, sometimes with severe cell damage to intestinal enterocytes and modulation of the gut bacterial community. The effect of dominance hierarchy formation, acute - and handling stress on the intestinal bacterial community have been reported in three salmonids; Arctic charr (*Salvelinus alpinus* L.), Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), and Atlantic cod (*Gadus morhua* L.). In Arctic charr, hierarchy formation reduced cultivable autochthonous (adherent) bacteria level in proximal intestine of subordinate fish compared to dominant fish, but this effect was not noticed in the distal intestine (DI). Furthermore, hierarchy formation modulates the gut microbiota. When Atlantic salmon and rainbow trout were exposed to acute stress the population level of adherent bacteria decrease in DI with concomitant increase in faeces, but this effect was not observed in Atlantic cod. The reason for the effect in Atlantic salmon is probably a peel-off effect of mucus and transporting autochthonous gut bacteria out of the fish. This will in turn allow allochthonous bacteria (present in the lumen or associated to digesta) to adhere and colonise the mucus layer. The elimination of the existing beneficial adherent gut microbiota and the lack of protecting mucus during acute stress might have relevance in pathogenesis. The present study reveals that stress eliminates certain protecting bacteria in the GI tract making the gut more prone to pathogen infections.

Key words: Fish, stress, intestinal microbiota.

INTRODUCTION

Stress in teleost fish is generally characterised by a set of changes in the normal metabolism. The stress response; thought to be compensatory and/or adaptive and enabling the animal to cope with stressors. Examples of stressors are; environmental changes (temperature and salinity), hauling, sorting, handling, transportation, pH stress and oxidative stress (Sandodden et al., 2001; Van Ham et al., 2003; Hoskonen and Pirhonen, 2006; Rollo et al., 2006; Torrecillas et al., 2012). Fish exposed to stress is known to have a major impact on; blood plasma (Biron and Benfey, 1994; Olsen et al., 2002; 2005; 2008), intestinal and enterocyte function; immediate damage to the junctional complexes evidenced by dissociation of both desmosomes and nexus (Peters, 1982; Szakolczai, 1997;

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Olsen et al., 2002; 2005), and increase the susceptibility to infectious diseases in fish (Wedemeyer, 1970; Snieszko, 1974; Mazeaud et al., 1977; Peters et al., 1988; Pickering and Pottinger, 1989).

In animals and humans, stress reduce the number of lactobacilli, beneficial bacteria, while there is an increase in epithelial adherence, prevalence and mucosal uptake of Gram-negative pathogens, for example, *Escherichia coli* and *Pseudomonas* (Tannock and Savage, 1974; Lutgendorff et al., 2008; Qiao et al., 2013), but the effect of different stress on the total bacteria level (CFU g^{-1}) and bacterial community in fish GI tract is less studied (Ringø et al., 1997; Ringø et al., 2000; Olsen et al., 2002, 2005, 2008). These studies revealed that fish exposed to stress affected both the total intestinal cultivable bacterial level and modulated the autochthonous (adherent) gut microbiota, those directly associated with the mucosa and mucus. Elucidation of the effect of stress on gut microbiota is of importance to evaluate as the elimination of the existing microbiota and loss of protecting mucus in stressed fish is likely to be important in pathogenesis of certain diseases that may use the GI tract as infection route (Groff and LaPatra, 2000; Birkbeck and Ringø, 2005; Ringø et al., 2010). If some of the existing bacteria present in the lumen are pathogenic, it is conceivable that they can adhere to membrane surfaces, result in mucosal tolerance or inflammation and translocate to infect otherwise sterile tissues and establish disease.

In addition to evaluation of studies published on the effect of stress on fish gut bacterial community, the present paper presents some data from unpublished studies having relevance with regard to the topic discussed.

EFFECT OF DOMINANCE HIERARCHY FORMATION ON GUT MICROBIOTA OF ARCTIC CHARR

Dominance hierarchy formation is a common feature of many fish species, and various aspects have been the subject of several studies (Sneddon et al., 2005; Paull et al., 2010) and is synonymous with access to food resources, shelter and/or reproduction. Within a hierarchy dominant individuals are more aggressive and grow markedly faster than subordinates, low-ranking individuals. To our knowledge, only one paper has been published on the effect of dominance hierarchy formation on fish gut microbiota (Ringø et al., 1997). In this study with Arctic charr, 373 autochthonous bacteria (aerobic and facultative aerobic) were isolated and enumerated from proximal intestine (PI) and distal intestine (DI) as described by Ringø (1993) and classified to genus level by standard biochemical tests as described by Muroga et al. (1987; Gram-negative bacteria) and Ringø (1993; Gram-positive bacteria). The study of Ringø et al. (1997) revealed that cultivable adherent bacterial level (CFU g^{-1}) in subordinate fish was reduced in the PI (log 3.48) compared to dominant fish; log 4.36. In contrast, no effect was noticed in the DI. Moreover, the composition of adherent bacteria was modulated as; *Aeromonas salmonicida* and *Xanthomonas* spp. were only detected in the GI tract of dominant fish, while bacteria belonging to the Cytophaga/ *Flexibacter* group were only detected in the digestive tract of subordinate fish. Furthermore, *Aeromonas hydrophila*, *Enterobacter* spp. *Vibrio fluvialis* and *Carnobacterium divergens* were only detected in DI of the subordinate fish and indicate that these bacteria genera are able to adhere and colonise the DI even when low feed intake occurs. Whether the modulation in the gut microbiota reported in this study affects the survival in challenge studies has not been elucidated and merits investigations.

EFFECT OF ACUTE STRESS ON GUT MICROBIOTA IN SALMONIDS

The intestinal mucus layer in fish has several important factors in fish health and welfare (Shepard, 1994). One important factor is the competitive exclusion of pathogens adhesion by intestinal bacteria (Ringø et al., 2005; Merrifield et al., 2014). Removal or disruption of the mucus layer therefore has the potential to open for pathogen adherence and loss of protective effect of the normal microbiota. In fish, the microbial communities have been shown to be essential for proper GI development and are implicit in aiding digestive function, immunological function and disease resistance (Gomez and Balcázar, 2008; Nayak, 2010).

In three studies with Atlantic salmon and rainbow trout evaluated the effect of stress on gut microbiota. Sampling of gut bacteria from control fish were carried out on fish not subjected to stress. The stress experiment was initiated by exposing the fish to acute stress by reducing the water level to 5-10 cm depth and subsequently chasing the fish with a pole for 15 min (Olsen et al., 2002; 2005; Zhou, He, Olsen and Ringø, unpublished data) and the gut microbiota were sampled according Ringø (1993); four hours post stress. The main effect of these stress studies was a peeling-off effect of mucus. In the study of Olsen et al. (2002) intestinal samples from mid intestine (MI), DI and faeces were collected for measurements of CFU g^{-1}. In fish subjected to stress, the population level of cultivable adherent gut bacteria (aerobic and facultative aerobic) in MI and DI were significantly reduced; from approximately log 4.2 prior to stress to ca. log 2.5 post stress, followed by a subsequent and significant increase in fecal CFU g^{-1}; from log 4.0 prior to stress to log 5.5 after stress. To confirm the results from cultivable bacterial analysis; electron microscopy evaluations of MI by methods described by Ringø et al. (2001) were carried out. Scanning electron microscopy evaluation; 4 h post stress revealed few bacteria at the enterocytes surface (Figure 1) compared to transmission

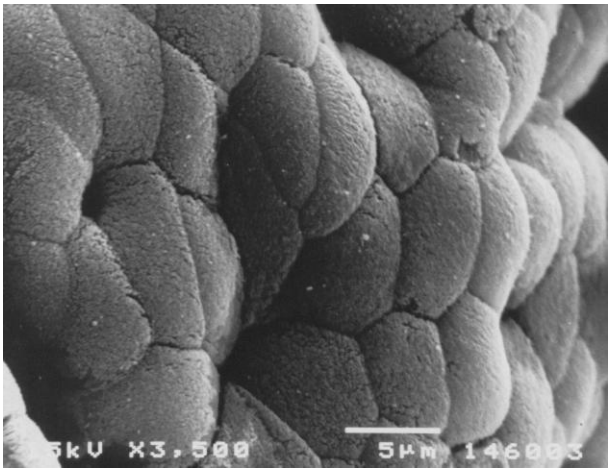

Figure 1. Scanning electron microscopy micrograph of the brush border membrane of midgut of Atlantic salmon 4 h after acute stress. Cell borders are clearly seen, but few bacteria are seen associated with the enterocytes surfaces. After Olsen, Myklebust and Ringø (unpublished data).

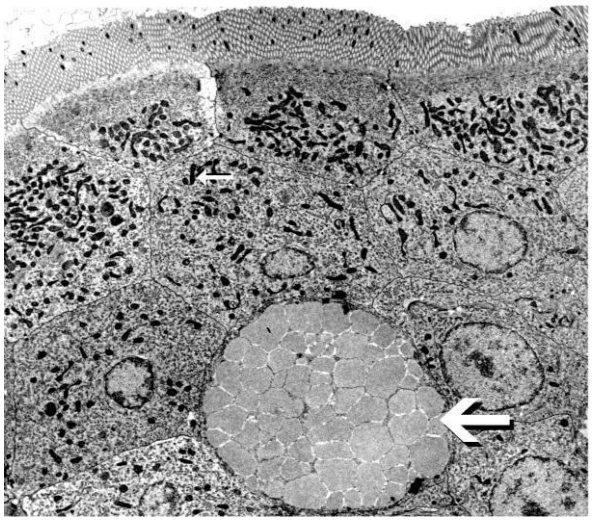

Figure 2. Transmission electron microscopy micrograph showing bacteria associated with the microvilli of enterocytes in the midgut of Atlantic salmon; not stressed fish. Small arrow - mitochondria; large arrow - goblet cell. After Ringø, Olsen and Myklebust and (unpublished data).

electron micrograph of a control fish where numerous bacteria are seen associated with the microvilli of enterocytes (Figure 2).

In the study of Olsen et al. (2005) totally 313 aerobic and facultative aerobic bacteria were isolated from unstressed and acute stressed rainbow trout. The population level of cultivable adherent aerobic and facultative aerobic bacteria (CFU g^{-1}) in DI decreased from log 4.3 (CFU g^{-1}) prior to stress to log 2.7 post stress, but increased in faeces from log 4.7 before stress to log 5.6 four hours post stress (Olsen et al., 2005). The concomitant bacterial increase in faeces post-stress revealed by Olsen et al. (2002, 2005) might have some relevance in the protection against other bacteria to adhere and colonise the intestinal mucosa. Furthermore, Olsen et al. (2005) reported elimination of some adherent bacteria genera in DI; *Acinetobacter* spp. and *Rhodococcus* spp., and a reduced level of *Pseudomonas* spp.; from log 3.79 to 2.62 CFU g^{-1}, and *Staphylococcus* spp.; from log 4.04 to log 1.10 CFU g^{-1}, prior to and post-stress, respectively. The increase in level of *Arthrobacter* and *Micrococcus* genera in faeces 4 h post-stress and their presence below detection level in the DI prior to stress might indicate that these bacteria genera either colonise the MI or they are weakness attached to the digestive tract mucosa and are lost during preparation; the intestines were rinsed three times in sterile 0.9% saline to remove non-adherent (allochthonous) bacteria.

A study devoted to evaluate adherent lactic acid bacteria (LAB) in the GI tract (PI, MI and DI) of fed Atlantic salmon exposed to regular (repeated) handling stress; reducing the water level to 5-10 cm depth and subsequently chasing the fish in the tank with a pole for 5 min (Ringø et al., 2000) revealed no significant reduction in LAB counts and LAB identified by phenotypic identification [Gram-staining, morphology, motility, production of catalase and oxidase, Huge and Leifson´s fermentation test, gas from glucose and growth on acetate agar (pH 5.4) and cresol red thallium acetate sucrose agar (pH 9.1)] and DNA sequence analysis in PI, MI or DI. In total 44, 36 and 35 strains were isolated from PI, MI and DI, respectively. Of these belonged 14, 8 and 8 isolates to LAB isolated from PI, MI and DI, respectively. As no further identification of gut bacteria not belonging to LAB was carried out; a further study evaluate the bacterial community in PI, MI and DI; sampled prior to stress and after 11 days of regular exposed to regular handling stress (Ringø, unpublished data). Removal of the GI tract from the fish is described in detail by Ringø (1993). Homogenates of the different gut segments were diluted in sterile saline solution and 100 µl of appropriate dilutions were spread onto the surface of two different tryptic soy agar plates as described by Ringø et al. (2000). Hundred and ninety-nine isolates were classified into 13 taxonomic groups by phenotypic identification as described by Ringø and Olsen (1999). A complex bacterial community was observed and included the Gram-negative genera; *Acinetobacter*, *Aeromonas*, *Alcaligenes*, Cytophaga/ *Flexibacter* - group, *Moraxella*, *Photobacterium*, *Pseudomonas*, *Xanthomonas* and the Gram-positive genera; *Brevibacterium*, *Microbacterium*, *Micrococcus*, LAB and *Staphylococcus*. These taxonomic groups were detected in PI, MI and DI prior to handling stress (Table 1). In contrast, after 11 days of regular handling stress only adherent *Acinetobacter*, *Pseudomonas*, *Xanthomonas* and LAB were isolated from the GI tract.

Table 1. Changes in log total viable counts (log TVC) of adherent cultivable bacteria genera present in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of Atlantic salmon (*Salmo salar* L.) prior to stress and after 11 days of regular stress. The log TVC values are means of six individual fish. After Ringø (unpublished data).

Bacteria	Prior to stress			11 days of regular stress		
	PI	MI	DI	PI	MI	DI
Log TVC	2.62	3.00	2.45	3.03	3.09	2.68
No. of isolates	44	36	35	25	28	31
Genus						
Acinetobacter spp.	1.76	1.74	1.51	2.63	2.60	2.14
Aeromonas spp.	1.76	n.d	n.d	n.d	n.d	n.d
Alcaligenes spp.	1.46	1.75	n.d	n.d	n.d	n.d
Cytophaga/*Flexibacter*-group	1.28	2.04	1.38	n.d	n.d	n.d
Moraxella spp.	n.d	n.d	0.90	n.d	n.d	n.d
Photobacterium spp.	n.d	1.44	n.d	n.d	n.d	n.d
Pseudomonas spp.	1.76	1.92	1.68	2.59	2.42	2.09
Xanthomonas spp.	n.d	1.74	1.51	n.d	2.49	1.89
Brevibacterium spp.	n.d	1.44	n.d	n.d	n.d	n.d
Microbacterium spp.	n.d	1.44	n.d	n.d	n.d	n.d
Micrococcus spp.	n.d	1.74	n.d	n.d	n.d	n.d
Staphylococcus spp.	1.76	2.40	1.68	n.d	n.d	n.d
LAB	2.13	2.35	1.81	2.24	2.24	2.03
Unknown*	0.98	1.44	1.38	1.64	1.94	1.49

11 days of regular stress had no significant effect on total viable counts of adherent bacteria in PI, MI and DI. n.d - not detected in the GI segment, LAB - lactic acid bacteria, * - died prior to positive identification.

This clearly showed that 11 days of regular handling stress modulates the autochthonous gut microbiota of Atlantic salmon. Based on these results we put forward the controversial hypothesis that the taxonomic groups; *Aeromonas*, *Alcaligenes*, Cytophaga/ *Flexibacter*, *Moraxella*, *Photobacterium*, *Brevibacterium*, *Microbacterium*, *Micrococcus*, and *Staphylococcus* are less strongly associated to the intestine and are lost during regular handling stress. This might open for opportunistic - and pathogenic bacteria to adhere and colonise the GI tract and initiate disease.

In the two salmonid studies of Olsen et al. (2002; 2005), the effect of stress on the intestinal microbiota was evaluated by conventional culture based methods. As conventional methods are time consuming and lack accuracy (Asfie et al., 2003) and sensitivity in characterizing certain fastidious and obligate anaerobes require special culture conditions, Zhou et al. (unpublished data) carried out an experiment where polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis was used to evaluate the bacterial community in acute stressed Atlantic salmon fed fish meal and fish oil. The autochthonous and allochthonous bacterial community in DI of three individual fish was evaluated by PCR-DGGE analysis according to Liu et al. (2008). The band position and its intensity were analysis by Glyko Bandscan 5.0 (Glyko, Novato, CA, USA), and the relative abundance (peak area) within the sample profile were calculated to the total bands gray-scale value. Cluster analysis was performed using the Jaccard's coefficient of similarity and the un-weighted pair group method (UPGMA) using the NTSYS software package. Obtained sequences were searched for in the GenBank library BLAST to find the closest relative for the partial 16S rRNA gene. Nucleotide sequences were deposit in GenBank under the accession Nos. EU697150 - EU697178. The results revealed higher values of pairwise similarity coefficient (Cs) in allochthonous bacterial community between the non-stressed and stress fish than the autochthonous bacterial community (Table 2), which indicate that autochthonous bacteria were more likely affected by acute stress than allochthonous bacteria. Representatives of the autochthonous and allochthonous bacterial communities of three not-stressed and three stressed fish are presented in Table 3, respectively. Stress stimulated abundance of some potential pathogenic bacteria, such as allochthonous *Lactobacillus letivazi* (AJ417738; band 19); not detected in non-stressed fish, autochthonous *Vibrio* sp. B5-8a (DQ357798; band 13), *Vibrio logei* strain T2110 (DQ318955; band 14 and 15), *Vibrio* sp. BC-R3 (DQ357806; band 16), and uncultured *Pseudomonas* sp. (DQ189764; band 28); not detected in non-stressed fish. Furthermore, a generally higher abundance of

Table 2. Pairwise similarity coefficient (Cs) matrix for the intestinal bacterial community of three individual Atlantic salmon fed fish meal and fish oil; not-stressed and acute stressed fish. DI - distal intestine. After Zhou et al. (unpublished data)

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
L1	1.0											
L2	0.8	1.0										
L3	0.8	0.67	1.0									
L4	0.87	0.93	0.67	1.0								
L5	0.87	0.73	0.93	0.73	1.0							
L6	0.8	0.73	0.83	0.73	0.83	1.0						
L7	0.87	0.73	0.93	0.73	1.0	0.83	1.0					
L8	0.8	0.73	0.8	0.8	0.73	0.73	0.73	1.0				
L9	0.76	0.7	0.63	0.7	0.7	0.7	0.7	0.57	1.0			
L10	0.83	0.77	0.7	0.83	0.77	0.7	0.77	0.9	0.6	1.0		
L11	0.83	0.77	0.7	0.83	0.77	0.7	0.77	0.9	0.6	1.0	1.0	
L12	0.83	0.77	0.7	0.83	0.77	0.7	0.77	0.9	0.6	1.0	1.0	1.0

Unstressed fish (control); L1, autochthonous microbiota from DI of fish no. 1; L2, allochthonous microbiota from DI of fish no. 1; L3, autochthonous microbiota from DI of fish no. 2; L4, allochthonous microbiota from DI of fish no. 2; L5, autochthonous microbiota from DI of fish no. 3; L6, allochthonous microbiota from DI of fish no. 3. Stressed fish: L7, autochthonous microbiota from DI of fish no. 1; L8, allochthonous microbiota from DI of fish no. 1; L9, autochthonous microbiota from DI of fish no. 2; L10, allochthonous microbiota from DI intestine of fish no. 2; L11, autochthonous microbiota from DI of fish no. 3; L12, allochthonous microbiota from DI of fish no. 3

Flavobacterium sp. SOC A4 (DQ628956; band 29) was observed in stressed fish. Whether the modulation of gut bacterial community has any effect on fish health and disease resistance is not known and merits further investigations.

IN VITRO GROWTH INHIBITION OF TWO FISH PATHOGENS BY CARNOBACTERIA ISOLATED FROM THE GI TRACT OF ATLANTIC SALMON PRIOR TO AND POST STRESS

Antibacterial activity by bacteria isolated from aquatic animals and the environment has been revealed in numerous studies (e.g. Gatesoupe, 1999; Gram et al., 1999; Sugita et al. 2002) as well as information about LAB isolated from the GI tract fish (Jöborn et al., 1999; Ringø et al., 2005; Ringø, 2008). The 199 *Carnobacterium* strains isolated and identified by phenotypic identification and DNA sequence analysis from the GI tract of Atlantic salmon; before stress, 5 h after stress, 24 h and 11 days following of regular handling stress by Ringø et al. (2000), where further tested for their ability to inhibit *in vitro* growth of two fish pathogens; *Aeromonas salmonicida* subsp. *salmonicida* LFI 4038 (furunculosis) and *Moritella viscosa* LFI 5000 (winter ulcer) (Ringø, unpublished data). *In vitro* growth inhibition was carried out using the microtitre method described elsewhere (Ringø et al., 2005; Salma et al., 2011; Askarian et al., 2012). The test revealed that 139 carnobacteria isolates inhibited *in vitro* growth of *A. salmonicida* while only 21 isolates inhibited growth of *M. viscosa* (Table 4). 97.4% of the bacterial strains isolated from fish prior to handling stress showed growth inhibition towards *A. salmonicida*, while only 76.7 and 46.9% of the isolated bacterial strains from fish; 5 and 24 h after stress, respectively displayed this ability. There were no significant differences between the different gut regions with regard to carnobacteria's antibacterial activity towards *A. salmonicida*. After 11 days of daily handling stress; only 43.2% of the carnobacteria isolated had the ability to inhibit *in vitro* growth of *A. salmonicida*. A similar trend as noticed in the growth inhibitions tests towards *A. salmonicida* was seen in the ability of carnobacteria to inhibit *in vitro* growth of *M. viscosa*. Prior to stress, approximately 16% of the isolates inhibited growth of *M. viscosa*, but after 11 days of regular stress only one out of 37 carnobacteria was able to inhibit growth of the pathogen (Table 4). The reason for the difference in the antibacterial activities of carnobacteria towards *A. salmonicida* and *M. viscosa* might be due to different infection routes. Previous studies have reported that *A. salmonicida* infect Atlantic salmon and rainbow trout via the intestine (Ringø et al., 2004; Jutfelt et al., 2006), while the skin is the possible infection route of *M. viscosa* as the bacterium was isolated from a natural outbreak of winter ulcer.

The reason why the frequency of carnobacteria able to inhibit the two pathogens decreased during the experiment is not known and call for further studies. One hypothesis might be that some carnobacteria revealing antibacterial activity are less strongly associated to the epithelial mucosa. The loss of the beneficial carno-bacteria strains colonising the intestine of Atlantic salmon may lead to adherence and colonisation of pathogens if they are present in the digestive tract when the stress is repeated over several days.

Table 3. Representative autochthonous and allochthonous bacterial community and their abundance in the DI of three individual Atlant ic salmon not exposed to stress. Auto - autochthonous; Allo - allochthonous. After Zhou et al. (unpublished data)

Not-stressed fish	Closest relative (Blast search)	Identity (%)	Accession no.	Fish no. 1		Fish no. 2		Fish no. 3	
				Auto	Allo	Auto	Allo	Auto	Allo
Band no.	**Bacteroides**								
3	*Flavobacterium psychrolimnae*	99	AJ585428	4.3		4.4		3.8	4.8
8	*Flavobacterium* sp. SOC A4(51)	98	DQ628945	7.3	5.8	16.8	7.2	14.9	6.4
29	*Flavobacterium* sp. SOC A4(51)	100	DQ628945	2.8	3.5	5.6	4.7	5.1	6.3
	Firmicutes								
5	*Lactobacillus aviarius* subsp. *aviarius*	98	AB326355	3.1	13.5		10.9		6.7
17	*Lactobacillus aviarius*	98	AB175728		11.4		17.5		8.7
18	*Lactobacillus aviarius* subsp. *aviarius*	100	AB326355	11.0	9.3		9.6		
11	*Anoxybacillus* sp.	96	DQ452025			3.8			14.5
6	Uncultured *Lachnospiraceae* bacterium	99	EF705084		7.2	1.6		10.3	
	Proteobacteria								
7	*Vibrio* sp. B5-8a	100	DQ357798	5.3	18.9		16.5		13.1
13	*Vibrio* sp. B5-8a	100	DQ357798		2.7				
10	*Vibrio logei* strain T21110	97	DQ318955			1.6			4.9
1	*Pseudomonas* sp. LV-5	100	EU580449	15.9	8.7	9.9	3.8	8.6	7.5
30	*Pseudomonas* sp. LV-2	98	EU580448	2.9	2.4	4.8	6.4	4.3	4.1
9	*Pseudomonas* sp. 65/3	100	EF513622	24.5	9.7	22.2	11.0	15.8	11.9
4	Gamma *Proteobacterium* RBE2CD-129	100	EF111261	5.9	7.6	16.2	11.9	12.5	8.7
	Unclassified bacteria								
2	Uncultured alpha *Proteobacterium*	85	AM237257	5.6		7.1		3.6	4.3
Stressed fish									
Band no	**Bacteroides**								
3	*Flavobacterium psychrolimnae*	99	AJ585428	1.8	4.1		6.3	5.2	3.9
8	*Flavobacterium* sp. SOC A4(51)	98	DQ628945	9.4	9.3	2.4	4.5	5.8	6.3
29	*Flavobacterium* sp. SOC A4(51)	100	DQ628945	8.9	6.7	4.5	6.3	7.3	4.6
	Firmicutes								
5	*Lactobacillus aviarius* subsp. *aviarius*	98	AB326355		9.5	1.0	6.9	3.9	9.5
17	*Lactobacillus aviarius*	98	AB175728		11.5		16.2	14.4	16.4
18	*Lactobacillus aviarius* subsp. *aviarius*	100	AB326355		7.9		7.2	13.9	11.4
19	*Lactobacillus letivazi*	95	AJ417738		5.2		13.8	4.7	7.5

Table 3. Contd.

11	*Anoxybacillus* sp.	96	DQ452025		4.8				
6	Uncultured *Lachnospiraceae* bacterium	99	EF705084	5.3					
	Proteobacteria								
7	*Vibrio* sp. B5-8a	100	DQ357798			9.7			
13	*Vibrio* sp. B5-8a	100	DQ357798			9.5			
16	*Vibrio* sp. BC-R3	98	DQ357806			18.4			
10	*Vibrio logei* strain T21110	97	DQ318955		2.3				
14	*Vibrio logei* strain T21110	99	DQ318955			8.6			
15	*Vibrio logei* strain T21110	98	DQ318955			6.3			
1	*Pseudomonas* sp. LV-5	100	EU580449	6.5	7.1	6.6	8.4	8.5	3.4
30	*Pseudomonas* sp. LV-2	98	EU580448	12.3	5.3	3.7	4.7	8.1	5.9
9	*Pseudomonas* sp. 65/3	100	EF513622	14.5	13.5	4.2	13.2	16.2	13.6
28	Uncultured *Pseudomonas* sp.	100	DQ189764				4.3	6.7	6.1
24	Uncultured *Photobacterium* sp.	100	AM936532			3.4			
4	Gamma *Proteobacterium* RBE2CD-129	100	EF111261	12.6	7.6	6.2	3.7	2.7	4.8
	Unclassified bacteria								
2	Uncultured alpha *Proteobacterium*	85	AM237257	8.1	5.6	3.5	4.5	4.5	7.3

Table 4. Growth inhibition of *Aeromonas salmonicida* LFI 4038 and *Moritella viscosa* LFI 5000 by carnobacteria isolated from three different regions of the GI tract of Atlantic salmon; prior to stress, 5 and 24 h after handling stress, and after 11 days of regular h andling stress. After Ringø (unpublished data). The growth inhibition test was carried out using the microtitre method described by Ringø et al. (2005), Salma et al. (2011) and Askarian et al. (2012).

Parameter	***Aeromonas salmonicida* LFI 4038**				***Moritella viscosa* LFI 5000**			
	A	**B**	**C**	**Sum**	**A**	**B**	**C**	**Sum**
Ultimately before	17/17	8/9	12/12	37/38	4/17	1/9	1/12	6/38
stress 5 h after stress	16/20	16/20	14/20	46/60	3/20	4/20	2/20	9/60
24 h after stress	11/21	13/20	16/23	30/64	1/21	3/20	1/23	5/64
11 days of regular stress	6/9	5/13	5/15	16/37	0/9	1/13	0/15	1/37
No. of carnobacteria able to inhibit the pathogen	50	42	47	139/199	8	9	4	21/199

Aeromonas salmonicida LFI 4038 was originally isolated from Atlantic salmon infected with furunculosis. *Moritella viscosa* LFI 5000 was originally isolated from Atlantic salmon from a natural outbreak of winter ulcer (NIFA, Tromsø, unpublished data). A - proximal intestine; B - mid intestine; C - distal intestine.

THE EFFECT OF ACUTE STRESS ON GUT MICROBIOTA IN ATLANTIC COD

Olsen et al. (2008) evaluated the effect of acute stress; similar to that used in the salmonid studies, in Atlantic cod. In general, the population level of adherent (autochthonous) cultivable bacteria (aerobic and facultative aerobic) ranged between 5.4 and 5.9 (log CFU g^{-1}) in cod intestines of undisturbed fish. Stress had no effect on the adherent bacterial population level in MI, but appeared to cause a small decrease, but non-significant, in both the hindgut (HG) and hindgut chamber (HC) sections. The fish did however seem to recover well, and by 48 h post stress there was a trend towards increased population level of cultivable gut bacteria in relation to initial levels in all groups (significant only for MI). The level of cultivable bacteria (log CFU) associated with digesta (allochthonous bacteria) from MI, HG and HC were 5.6, 5.6 and 5.9 CFU g^{-1}, respectively in undisturbed fish. A minor reduction was observed in MI 4 h after stress (log TVC = 5.1), but not revealed in the other sections. However, 48 h post stress, there was an increase in cultivable allochthonous bacteria in HG and HC with the most pronounced effect observed in HC (log 6.7 CFU g^{-1}). A total of 360 cultivable bacterial strains were isolated from the GI segments, before and 48 h post stress. Bacteria belonging to *Carnobacterium* dominated before stress and accounted for 63.3% of the cultivable bacteria. Of these were 22 strains identified as *Carnobacterium maltaromaticum* by partial sequence of 16S rRNA gene. However, subjecting Atlantic cod to stress reduced the proportion of *Carnobacterium* to approximately 23% of total cultivable bacteria. These data show many similarities to the salmonid studies referred above as the protective bacteria are eliminated following stress. This result might have relevance in the potential to retard pathogenic colonisation of the epithelial mucus layer within the intestine of fish, which is important because adhesion to epithelial mucus is thought to be an important step in intestinal infection, providing a foundation for mucosal interaction and translocation (Ringø, 2004; Ringø et al., 2010; Sica et al., 2012; Merrifield et al., 2014).

Isolates belonging to *Photobacterium phosphoreum* and *Photobacterium* spp. - like isolates were detected as allochthonous in MI of unstressed fish, while in fish 48 h post stress similar bacteria were isolated from HG (allochthonous) and HC; as autochthonous bacteria. Adherent *Pantoea agglomerans* and *Pantoea* spp. - like isolates were detected only in the MI of fish; 48 h after stress. In MI of unstressed fish, *Vibrio logei* and *Vibrio* spp. - like strains were isolated, while strains showing high similarity (98%) to an uncultured *Vibrio* sp. clone C7 were isolated in HG of stressed fish. *Bacillus* spp. was detected associated with digesta from the HG of unstressed fish, but was not detected in stressed fish. *Enterobacter* was detected in the gut of stressed fish. In contrast, *Enterobacter* strains were not detected in any sample prior to stress. Differences between unstressed and stressed fish can further be illustrated by the fact that strains showing high similarity to uncultured bacterium clone E792 and uncultured bacterium clone AKIW6008 were only detected in the gut of unstressed fish, but then as allochthonous bacteria present in HG and HC.

The peel-off mucus effect observed in salmonids (Olsen et al., 2002; 2005) was not observed in the Atlantic cod study and these results were supported by Ussing chamber data from four fish per treatment where the transepithelial resistance increased with time suggesting a higher mucus production and that this mucus is attached to the intestinal mucosa (Olsen et al., 2008).

PROBIOTICS AND STRESS

Currently there is a growing interest in the use of probiotics in aquaculture (Lauzon et al., 2014; Newaj-Fyzul et al., 2013). Mohapatra et al. (2013) discussed different aspects of stress management and probiotic intervention, but as no information is reported on the role of probiotics in fish related to different environmental stress and their effect on gut microbiota and fish health, we recommend that the topic should be given high priority in the future.

When discussing the use of probiotics in aquaculture and the antagonistic effect of gut bacteria towards pathogens, it is worth to mention that in a probiotic study with Atlantic salmon, Gram et al. (2001) used *Pseudomonas fluorescens* strain AH2; a strain showing strong *in vitro* inhibitory activity towards *A. salmonicida* (Gram et al., 1999). However, co-habitant infection by *A. salmonicida* in Atlantic salmon did not result in any effect on furunculosis-related mortality (Gram et al., 2001). Based on their results, the authors concluded that a strong *in vitro* growth inhibition cannot be used to predict a possible *in vivo* effect.

CONCLUSIONS AND FURTHER PERSPECTIVES

One of the most important goals for fish microbiologists has been to obtain a stabile indigenous gut microbiota of fish. However, hierarchy formation, acute stress and handling stress, the microbial balance become disturbed and disordered, and the complex relationship of the indigenous gut microbiota with the host can be depicted in a multilevel framework; where luminal bacteria, the mucus layer and the innate and adaptive immunity interact.

The practical effect of this activity is the exclusion of beneficial bacteria which may result in adherence and colonisation of invading populations of nonindigenous microorganisms, including pathogens in the GI tract in fish. The antagonistic effect towards pathogens by the

gut microbiota is possibly mediated by competition for nutrients and adhesion sites, formation of metabolites such as organic acids, hydrogen peroxide, and production of antibiotic-like compounds and bacteriocins. A fundamental question arises when discussing the protective role of the GI microbiota; is the antagonistic gut microbiota affected by stress and do changes in the gut microbiota have any negative health effect? This has to be elucidated in future studies.

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