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# Effect of *Anisakis simplex* (*sl*) larvae on the spoilage rate and shelf-life of fish mince products under laboratory conditions

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ABSTRACT

Wild caught marine fish are commonly infected with anisakid nematodes lodging in the intestinal linings or in the fish muscle. One of the most commonly found nematode parasites in marine fish is *Anisakis simplex*. During production of mince from the muscle of wild caught *Anisakis*-infected fish, the larvae would be disrupted during mince production. Any bacteria within or on the surface of such larvae are during the mincing process evenly distributed throughout the mince, and could thus possibly affect the spoilage rate of the final products. To explore if or how any bacteria associated with muscle-invading *Anisakis* larvae may affect the spoilage rate of fish mince, a controlled storage trial was conducted. Fillets of farmed Atlantic cod (*Gadus morhua*), exclusively fed on dried and heat-treated compound feed and hence expectably free from *Anisakis* larvae, were aseptically collected and homogenised. Fish mince aliquots were added different volumes of *Anisakis* homogenate based on larvae which were freshly sampled from the visceral cavity of NE Atlantic blue whiting (*Micromesistius poutassou*). The volumes of added parasite homogenate (parasite(+)-samples) reflected different infection intensities from 15 (low) to 50 (high) larvae per 100 g fish fillet, representing an actual *Anisakis* intensity range in the flesh of blue whiting. The samples were kept at 4 °C for 15 days and subjected to microbiological, sensory and chemical evaluation at 3 days intervals. Upon visual examination and plate count measurements (PC) on Iron Agar Lyngby (IAL), the samples without any parasite additives (no[parasite]) spoiled differently and more rapidly than any of the parasite(+)-samples. However, $H_2S$-producing bacteria were only recorded in the latter samples, which were also the only ones that showed increased levels of the spoilage indicator substance trimethylamine (TMA). Moreover, the parasite(+)-samples changed their sensory characteristics at a later stage compared to the no[parasite]-samples. Although some cultures of $H_2S$-producing bacteria were found on IAL, molecular identification by PCR-DGGE of the actual bacteria was not conclusive. *Psychrobacter* sp. which has no or only little spoilage activity, was identified in all samples until trial day 9, but was probably outgrown by the stronger spoilers *Pseudomonas fluorescence/fragi* and *Photobacterium phosphoreum*. Thus, and somewhat unexpected, our findings indicate that – under the present trial conditions – fish mince contaminated with bacteria which originate from *Anisakis* larvae, spoiled less rapidly than samples without any parasite-related bacteria present. Moreover, the shelf-life of fish mince was apparently not reduced by the presence of bacteria transferred to the mince by *Anisakis* larvae.



## 1. Introduction

Many global wild living fish resources are limited and an over-exploitation of several species can be seen. Utilising new fish species for human consumption is therefore an important step to comply with the high demand for high quality protein. NE Atlantic blue whiting (*Micromesistius poutassou*) has for several years been an important resource for the fish meal industry, partly because catches of this stock are often unfit for human consumption due to the notorious infection with the larvae of the parasitic nematode *Anisakis simplex*. According to Levsen, Lunestad, & Berland (2005), the *Anisakis* infection intensity in the flesh of blue whiting typically ranges from 0 to 71, with mean values of 10.7 ± 14.1 larvae per fillet. Besides the quality reducing effect of *Anisakis* larvae – encountering worms in a given fish product is most repellent – they are of human health concern due to the potential to cause anisakiasis, *i.e.*

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direct infection with live larvae (Jay, Loessner, & Golden, 2005). *A. simplex* is present in many commercially utilised wild fish species but is killed by proper freezing or heat treatment. The current rules and regulations state that all wild caught fish species, if to be eaten raw or almost raw, must be frozen at − 20 °C for at least 24 h (Codex Committee on Fish and Fishery Products, 2004; European Commission, 2004). In order to still using NE Atlantic blue whiting as a food resource but avoiding any problems inflicted by the *Anisakis*, the fish could be used as raw material in the production of fish mince or surimi (Trondsen, 1998). The quality reducing effect associated with *A. simplex* larvae in fish flesh has previously been described by us (Svanevik, Levsen, & Lunestad, 2013). We found the intestine of *Anisakis*-larvae to be densely populated with bacteria including the specific fish spoilage bacteria *Photobacterium phosphoreum* and *Shewanella* sp., which may possibly influence the microbial product quality if introduced into the fillets by migrating *A. simplex* larvae.

*P. phosphoreum* is recognised as the dominating spoilage bacteria of fish products packed in modified atmosphere (MAP) and stored at low temperatures (Dalgaard, Gram, & Huss, 1993; Dalgaard, Mejholm, Christiansen, & Huss, 1997; Hovda, Lunestad, Sivertsvik, & Rosnes, 2007). The bacteria utilise trimethylamine oxide (TMAO) (Gram & Huss, 1996), which is naturally found in fish, by reducing it to trimethylamine (TMA) which again has the recognisable odour of degraded fish (Huss, 1994). Species of *Shewanella*, in particular *Shewanella putrefaciens*, are also known as spoilage contributors through the production of hydrogen sulphide ($H_2S$) (Gram & Dalgaard, 2002; Gram & Huss, 1996; Jørgensen & Huss, 1989; Vogel, Venkateswaran, Satomi, & Gram, 2005). Analysis for trimethylamine-nitrogen (TMA/N) is commonly used as a spoilage indicator for fish and fish products.

Other members of the cod family (Gadidae) *e.g.* Atlantic cod (*Gadus morhua*) and saithe (*Pollachius virens*) are also acting as host for *Anisakis* larvae (Strømnes & Andersen, 1998). These fish species have comparable composition of fillet macro nutrients, *i.e.* low fat (0.3–0.8 g 100 $g^{-1}$) and carbohydrate (<0.1 g 100 $g^{-1}$) content, and higher protein content (17.8–20.0 g 100 $g^{-1}$) (DTU Food, 2014; National Institute of Nutrition and Seafood Research, 2014), and are often used in private households or industrial fish mince production. During fish mince production, *Anisakis*-infected fillets are homogenised and any larvae present will be disrupted and subsequently distributed throughout the mince. Thus, based on our previous findings (Svanevik et al., 2013), the main objective of the present study was to experimentally examine the effect of bacteria associated with fish muscle-invading *Anisakis* larvae on the microbiota of fish mince products, and hence the shelf-life of the final product. Additionally, we aimed to investigate the relationship between larval infection intensity, *i.e.* the number of *Anisakis* larvae in the fish flesh, and the spoilage rate of the product. The study was designed in order to simulate an authentic *Anisakis* infection situation in the industrial production of fish mince based on fillets of NE Atlantic blue whiting.

## 2. Materials and methods

### 2.1. Fish mince

To ensure that the fish mince used in this study was initially free from any *Anisakis* larvae, a substitute for blue whiting fish mince was made from the flesh of freshly harvested farmed Atlantic cod (*G. morhua*). The latter are exclusively fed formulated feed, and are therefore not likely infected with *Anisakis* larvae. From the cod, caudal muscle blocks without skin were aseptically collected and transferred to a food processor metal bowl, added sterile $H_2O$ (50 ml $H_2O$ per kg fish) and homogenised for 2 min (Dito K35, Electrolux). The homogenate was kept in 100 g portions in sterile plastic bags at −20 °C until further analysis.

### 2.2. Parasite homogenate

Encapsulated *A. simplex* larvae were collected from the visceral cavity of freshly caught blue whiting (*M. poutassou*). The fish were opened by using a sterile scalpel, and the larvae were removed from the host by sterile tweezers. After removal of the host induced connective tissue capsule, the larvae were counted and kept in sterile plastic tubes. A total of 500 larvae were diluted in 20 ml sterile peptone water and homogenised with a hand held mixer (Polytron PT 1200 Cl) for 2 min, yielding a concentration of 25 *Anisakis* larvae per ml. The bacterial plate count was enumerated as described in Section 2.4. The parasite homogenate was stored at 4 °C prior to analysis.

### 2.3. Inoculation and storage

The frozen fish mince bags were thawed at 4 °C over night (16 h) and kept on ice during sample preparation. Three series were prepared, each consisting of four fish mince bags added different volumes of parasite homogenate, *i.e.* 0.0 ml, 0.6 ml, 1.2 ml and 2.0 ml corresponding to 0, 15, 30 and 50 larvae per 100 g fish mince, respectively. The samples will further be referred to as no [parasite], low[parasite], medium[parasite] and high[parasite]. All fish mince bags were subsequently stored in an incubator (Thermo Scientific) at 4 °C throughout the trial period (15 days). Finally, two fish mince bags without any parasite homogenate additives were incubated as the above set of samples, but were not analysed before trial end at day 15. These samples are referred to as negative controls.

### 2.4. Cultivation

Aliquots of the 3 different concentrations of parasite homogenate were spread on the surface of Iron Agar Lyngby (IAL) and incubated at 20 °C for 72 h to calculate the bacterial concentration. Unique colonies from these plates were grown into pure culture by three times transfer onto IAL, collected into individual Eppendorf tubes for further molecular identification These are referred further on to as "pure culture" samples. During the storage trial, the fish mince bags were analysed at 3 days intervals starting at day 0 before and after inoculation and further at day 3, 6, 9, 12 and 15. At every sampling, 5 g of matrix were aseptically collected from each mince bag and transferred into small stomacher bags, diluted 1:10 in peptone water and homogenised for 30 s in a Stomacher micro-biomaster (Seward). From the homogenate, a dilution series was made and appropriate aliquots were spread on the surface of IAL. All plates were incubated at 20 °C for 72 h before enumeration of black colonies ($H_2S$-producing bacteria) and total plate counts (PC). The bacterial content of plates with countable numbers from each sampling *i.e.* 4 samples at day 0 before inoculation, 3 samples at day 0 after inoculation, 4 sample at day 3, at day 6, at day 9, at day 12, at day 15, plus a mix of 2 control samples at day 15, were collected into separate Eppendorf tubes thus representing "bulk cells" as described by Svanevik et al. (2013).

### 2.5. Characteristical changes

Sensory observations included changes in texture and colour, as well as odour characteristics, and were recorded by the same person throughout the experiment.

### 2.6. Chemical marker of spoiling activity and pH

After production of the fish mince, two samples of 30 g each were transferred into plastic containers and stored at −20 °C. During the storage trial, 10 g of each fish mince bag was pooled with the corresponding triplicate and the pH was measured at day 3, 9, 12 and 15. All pooled samples were stored at −20 °C and analysed for trimethylamine-nitrogen (TMA/N) soon after the trial ended. This was done by the titration method as described by Conway & Byrne (1936).

### 2.7. Molecular identification

DNA from all "pure cultures" and "bulk cells" was extracted and purified before amplification by the same PCR method. Fragments from "pure cultures" were sequenced directly by Sanger sequencing, whereas the "bulk cells" from the storage trial were run on denaturing gradient gel electrophoresis (DGGE) to separate the different bacterial fragments prior to sequencing. All analyses were performed as described by Svanevik and Lunestad (2011), except of the PCR enzyme where GoTaq® DNA Polymerase (Promega) were used instead.

### 2.8. Statistics

A one-way ANOVA, followed by a Tukey HSD *post hoc* test, was run in order to determine the significance level of the differences in mean values of the PC and $H_2S$-producers among all four sample series. Prior to analysis, all data were log-transformed ($\log(x+1)$). The dependent variable, log CFU $g^{-1}$, was categorised according to parasite concentration and days after trial onset. The significance level was set at 0.05. All statistics was done in Statistica 11 (StatSoft, Inc 2012), while graphs were produced in GraphPad Prism 6 (GraphPad Software, Inc).

## 3. Results

### 3.1. Cultivation

The initial parasite homogenate used for inoculation contained 4.4 log CFU $ml^{-1}$, while the number of bacteria added to the fish mince bags (100 g) was 4.2 log CFU for low[parasite], 4.5 log CFU for medium[parasite] and 4.7 log CFU for high[parasite]. In plate counts (PC) of all samples during the storage trial (Fig. 1), significant differences were found between the mean values of no[parasite] and low[parasite] at day 3, 9, 12 and 15 ($p < 0.05$), and between no [parasite] and medium- and high[parasite] at day 9, 12 and 15 ($p < 0.05$). Among the $H_2S$-producing bacteria from all samples during the storage trial (Fig. 2), significant differences ($p < 0.05$) were found at every intermittent sampling (3 days intervals) between all three parasite(+)-samples, and no[parasite]. Additionally, significant differences were recorded between low[parasite] and high[parasite] at day 6.

### 3.2. Characteristic changes

At day 0, the fish mince in all samples appeared as semi-dry white and with a texture and odour characterising a fresh fish product. The first changes were recorded at day 9 in no[parasite] samples which appeared slightly viscous with a greenish mucous layer and a sweetish odour. Similar registrations were made in the same samples at day 12, although with increased sensoric intensity. The negative control samples which were examined at trial end (day 15) only, had spoiled in the same manner. At day 15, in one of the low[parasite] samples also a greenish mucous layer was seen. Sensory changes in the medium- and high[parasite] samples appeared not until trial end at day 15 when the samples were slightly dryer and a $H_2S$ odour was evident.

### 3.3. Chemical spoilage indicator and pH

The pH of all samples dropped until trial day 9, however, only the parasite(+)-samples went below pH 6. While all samples had fairly the same initial pH (day 3), the no[parasite] samples did not drop significantly and also showed the highest values at all measure points (days 9, 12 and 15) (Fig. 3). The analyses for TMA/N revealed that the parasite(+)-samples reached the highest concentrations after 9 days, with the steepest increase between trial onset and day 3, except of the low[parasite] samples which increased most rapidly between trial days 3 and 9. However, the TMA/N remained very low and practically constant in the no [parasite] samples throughout the trial (Fig. 4), thus the negative control samples had some elevated levels.

### 3.4. Molecular identification

As recommended by Venter et al. (Venter et al., 2004) sequence similarities that had a GenBank Blast match ≥ 97%, were accepted as taxon identification. Among the "pure cultures" samples isolated

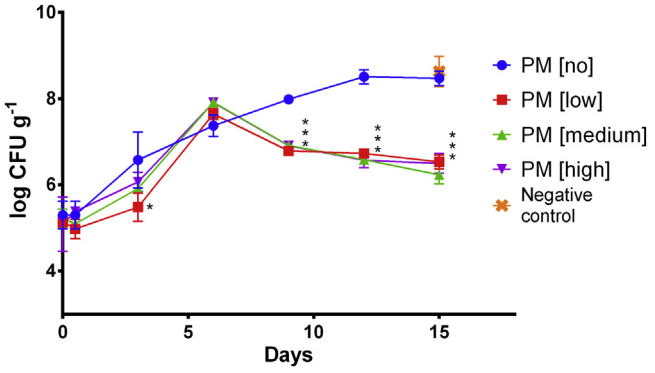


**Fig. 1.** Mean plate counts (PC) (log (CFU $g^{-1}$ + 1)) ± SD in samples with no, low, medium and high[parasite]. The additional negative control samples were only analysed at day 15. All samples were cultured on IAL and incubated at 20 °C for 72 h * differs significantly (<0.05) from "no[parasite]".

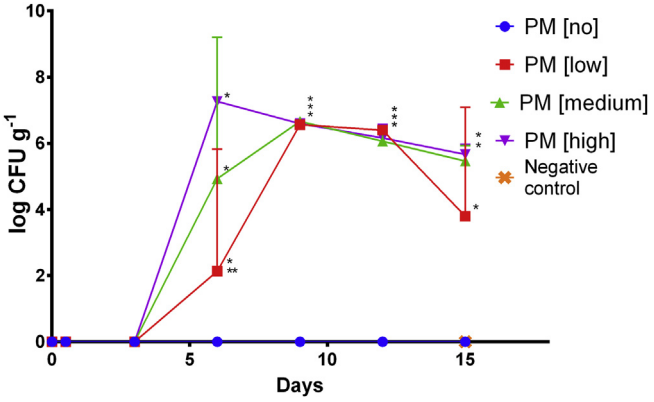


**Fig. 2.** Mean counts of $H_2S$-producing bacteria (log (CFU $g^{-1}$ + 1)) ± SD in samples with no, low, medium and high[parasite]. The additional negative control samples were only analysed at day 15. All samples were cultured on IAL and incubated at 20 °C for 72 h *differs significantly (<0.05) from "no[parasite]". **differs significantly (<0.05) from high[parasite].

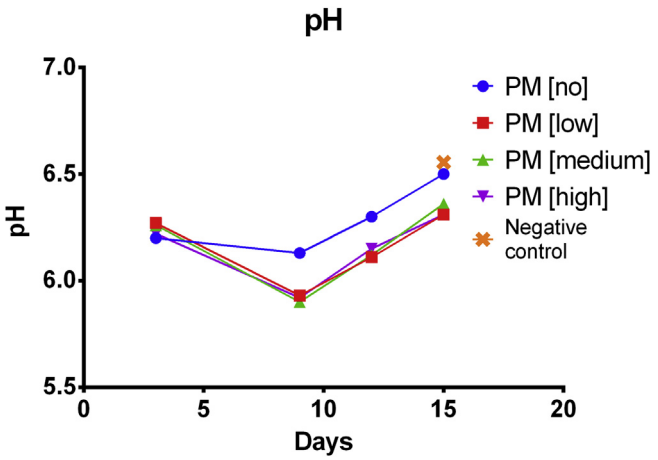


**Fig. 3.** pH measurement of pooled parallel samples of no, low, medium and high [parasite]. The additional negative control samples were only analysed at day 15.

from the parasite homogenate plates, 16 samples were identified as *Aequorivita* sp. (99% GU570646), *Arenibacter latericius* (100% JQ898116), Bacteroidetes bacterium (99% AY922251), *Gelidibacter mesophilus* (99% AJ344134), Microbacteriaceae bacterium (100% JQ259582), *P. phosphoreum* (99% AB681911), *Pseudoalteromonas* sp. (99% JX407138/JQ342687), *Pseudomonas fluorescens* (97% EF408245/JX090149), *Pseudomonas* sp. (99% JQ229609/FJ013347), *Psychrobacter celer* (99% JF711007), *Psychrobacter* sp. (99% JN602224) and *Pusillimonas* sp. (98% EU734657).

PCR-DGGE of the "bulk cell" samples resulted in 30 bands of which 18 bands were isolated (Fig. 5) and sequenced. Three different taxa were identified *i.e. Psychrobacter* sp. (100% JQ991576), *Pseudomonas fragi/fluorescens* (100/99% AB685609/EF408245) and *P. phosphoreum* (97% AB681911). The bacteria taxa present per sample type are shown in Table 1.

## 4. Discussion

The present study aimed primarily to investigate experimentally if or how the presence of bacteria associated with *Anisakis* larvae would affect the microbiota in fish mince, with special emphasis on the specific spoilage bacteria. The culture medium chosen was Iron Agar Lyngby (IAL), as recommended in several studies which showed increased retrieval and growth of seafood associated bacteria on IAL compared to other common culture media (Broekaert, Heyndrickx, Herman, Devlieghere, & Vlaemynck, 2011; Gram, 1992; Gram, Trolle, & Huss, 1987; Parlapani, Meziti, Kormas, & Boziaris, 2013). The mean plate counts for the different fish mince samples showed that no[parasite] had increasing numbers until flattening at day 12, reaching maximum bacterial numbers of 8.4 log CFU $g^{-1}$ (Fig. 1). Similar results have been reported for several other fish species kept under comparable storage conditions (Broekaert et al., 2011; Gram & Huss, 1996; Gram et al., 1987; Parlapani et al., 2013). The bacterial growth of the parasite(+)-samples increased during the first six days followed by a lesser decline during the remaining trial period. No significant differences in CFU $g^{-1}$ were found among the parasite(+)-samples, which indicates that there was no direct relationship between bacterial growth and the larval infection intensity in the fish mince (*i.e.* number of *Anisakis* larvae per 100 g fillet) under the present trial conditions. However, the significant differences in CFU $g^{-1}$ which we recorded at day 9, 12 and 15 between no[parasite] and the three parasite(+)-samples imply that, in this case, the addition of parasite homogenate restricted bacterial growth. This was the opposite effect of what we expected.

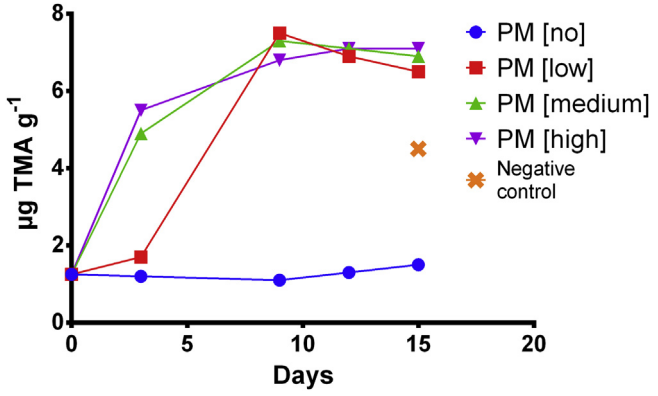


**Fig. 4.** TMA analysis (mg TMA/N 100 $g^{-1}$) of pooled parallel samples of no, low, medium and high[parasite]. The additional negative control samples were only analysed at day 15.

Parlapani et al. (2013) reported that the proportion of $H_2S$-producing bacteria in fish products increases during storage, by numbers eventually reaching the plate count (PC). This assumption does not comply with the findings of the present study, since no $H_2S$-producing bacteria were found on plates from the no[parasite] samples. However, $H_2S$-producing bacteria were recorded on the plates from the parasite(+)-samples. The high[parasite] increased most rapidly towards day 6 reaching 7.3 log CFU $g^{-1}$, followed by medium[parasite] at 4.9 log CFU $g^{-1}$, and low[parasite] at 2.1 log CFU $g^{-1}$ (Fig. 2). However, significant differences were found only between low[parasite] and high[parasite] at day 6. This may indicate that the initial growth rate of $H_2S$-producing bacteria depended on larval concentration in the samples, and hence, that the mince containing high[parasite]could spoil more rapidly.

The trimethylamine-nitrogen (TMA/N) measurements corresponded well with the results from the $H_2S$-producing bacteria. No [parasite] had a stable low concentration (~1.25 mg TMA/N 100 $g^{-1}$) throughout the storage, indicating very little growth, or even absence, of TMA-producing bacteria. The same trend as for $H_2S$-producing bacteria was seen among the parasite(+)-samples, with values ranging 6–7 mg TMA/N 100 $g^{-1}$ in all samples on day 9 and throughout the trial. These findings show that a major proportion of TMA-producing bacteria were in fact introduced into the fish mince by the *Anisakis* larvae. Other TMA/N studies of fresh cod muscle found that TMA/N values often are below 1 mg TMA/N 100 $g^{-1}$ after five days storage on ice, but may increase to approximately 5 mg TMA/N 100 $g^{-1}$ after 11 days, and further to 10–20 mg TMA/N 100 $g^{-1}$ after 15 days (Baker, Ranken, & Kil, 1997). The TMA/N concentration at which to reject a given fish product for consumption could be hard to determine. However, Lynum (1994) states that a fresh Gadidae fish should hold less than 1.5 mg TMA/N 100 $g^{-1}$, and upon rejection the TMA/N content would range between 8 and 15 mg 100 $g^{-1}$. The negative control samples showed higher TMA/N values (4 mg 100 $g^{-1}$) than the no[parasite] samples, but still lower than the *Anisakis* (+)-samples. These samples were not analysed until day 15, and were kept closed and unmixed until analysis start. All the other samples were exposed to air during mixing before each sampling every third day. Some bacteria, including certain *Pseudomonas* spp., use TMAO as terminal electron acceptor whenever oxygen levels are low, producing TMA (Debevere & Boskou, 1996). This may explain the higher TMA/N concentrations of the unaerated negative control samples compared to the no[parasite].

Although no $H_2S$-producing bacteria were found in no[parasite], the sensoric changes in the samples, characterised by a sweetish off-odour and a greenish mucoid surface layer, indicate that they

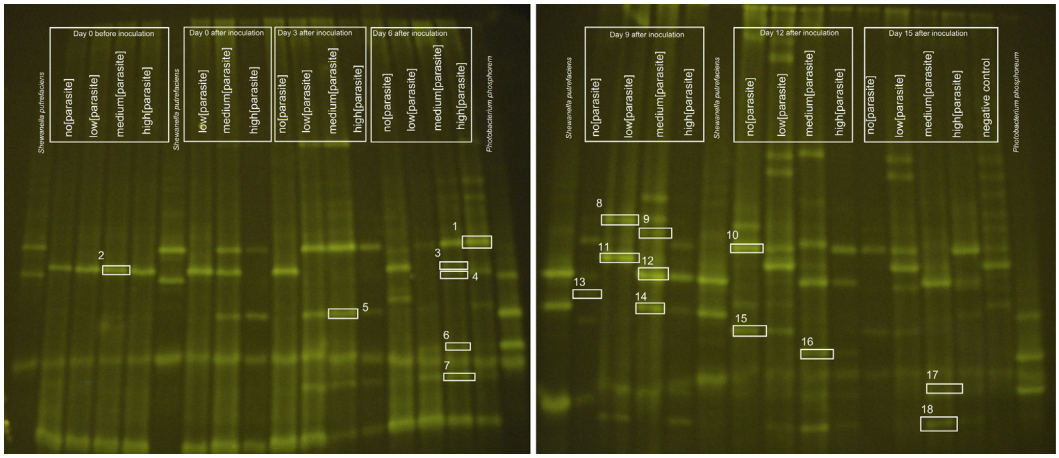


**Fig. 5.** The DGGE profile of a fish mince samples stored for 15 days. Excised bands that were sequenced (No. 1-18), where No. 1, 2, 3, 4, 5, 12, 14 and 16 were identified as *Psychrobacter* sp. (JQ991576). No. 6, 8, 10, 11, 13, 15 and 17 were identified as *Pseudomonas fragi/fluorescens* (AB685609/EF408245), whereas No. 7, 9 and 18 were identified as *P. phosphoreum* (AB681911). *S. putrefaciens* and *P. phosphoreum* were run as external standards.

spoiled more rapidly. Thus, the changes seemed to be induced by other species than those commonly associated with the production of $H_2S$ and TMA. The pH of the parasite(+)-samples decreased from 6.2 at day 0 to 5.9 at day 9, before rising to 6.1 and 6.3 at day 12 and 15, respectively. The fact that the no[parasite] samples were always above pH 6.0, with generally slightly higher values than all other samples throughout the trial may indicate that the bacteria in the former samples faced somewhat better growth conditions. Different fish spoiling bacteria have different carbohydrate metabolisms which again may affect the pH of the actual medium. Thus, specific metabolic properties could account for the slightly lower pH in the parasite(+)-samples and, therefore, the inhibition and decline in bacterial growth in these samples since fish spoiling bacteria are known to grow better at pH > 6.0 (Adams & Moss, 2008).

The identification of bacterial taxa in the parasite homogenate showed that *Anisakis*-larvae may act as a vector for bacteria from the fish intestine into the fish flesh. However, many of the species that were identified in the parasite homogenate were not recovered from the parasite inoculated fish mince samples by the PCR-DGGE method. *Psychrobacter* sp. and *P. fragi/fluorescens* were found in samples both with and without parasite homogenate added, whereas *P. phosphoreum* were found exclusively in the parasite(+)-samples. It is well documented that *P. phosphoreum* contributes to off-odour spoilage involving production of TMA and other degradation products in fish from northern temperate waters stored at refrigerator temperature, but especially in fish that is packed in modified atmosphere (MAP) (Dalgaard et al., 1997; Gram & Dalgaard, 2002; Hovda et al., 2007). *P. phosphoreum* does not produce $H_2S$ (Dalgaard et al., 1993) and neither does *P. fluorescens*, *P. fragi* or any *Psychrobacter* species (Gennari, Tomaselli, & Cotrona, 1999). Probably due to sensitivity constraints, PCR-DGGE analyses came out with the same probability for the presence of *P. fluorescens* and *P. fragi,* although each possesses different properties as active spoiler. For example, *P. fluorescens* is a commonly occurring fish spoiler that produces TMA and could thus be a significant TMA-contributor, together with *P. phosphoreum,* in the parasite(+)-samples (Gram et al., 2002). *P. fragi* is mainly associated with a sweetish and fruity odour due to production of ethyl esters, and could thus be responsible for the characteristic sweetish spoilage odour in no[parasite]-samples (Miller, Scanlan, Lee, & Libbey, 1973). Since PCR-DGGE probably cannot differentiate between the two species, the same *Pseudomonas* species may have been present in either sample type. Members of the genus *Psychrobacter* are known to contribute to no or minor spoilage (Gennari et al., 1999), and it seems that this species is outgrown by other stronger specific spoilers in those samples that spoiled most rapidly, *i.e.* the no[parasite]- and low[parasite]-samples. *Psychrobacter* sp., *P. fragi/fluorescens* and *P. phosphoreum* were recorded in the medium[parasite] samples at day 12, and in the high[parasite]-samples at day 9, 12 and 15. Thus, interactions among these bacteria could, together with the lowered pH, account for the decline in PC after day 9. Gram et al. (2002) reported that interactions between spoilage bacteria could have antagonistic effect, and thus be important for the bacterial growth in food products. Compared to the no[parasite]-samples, where only one species was identified at each sampling, the medium- and high[parasite]-samples showed a more complex bacterial composition (Table 1).

The most common spoiling- and $H_2S$-producing bacteria of chilled fish stored aerobically, *Shewanella putrefaciens* (Gram & Huss, 1996), was not identified by molecular methods in the present study, neither were any other $H_2S$-producing species, suggesting that these active spoilers were absent. However, this could not have been the case since we recorded typical colonies with

**Table 1**
Presence of *Psychrobacter* sp., *Pseudomonas* sp. and *Photobacterium phosphoreum* in fish mince samples over time.

| | Day 0[a] | | | | Day 0[b] | | | Day 3 | | | | Day 6 | | | | Day 9 | | | | Day 12 | | | | Day 15 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterium/[parasites] | N | L | M | H | L | M | H | N | L | M | H | N | L | M | H | N | L | M | H | N | L | M | H | N | L | M | H | C |
| *Psychrobacter* sp. | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | | X | X | | | X | X | | | X | X | |
| *Pseudomonas* sp. | | | | | | | | | | | | | | | | X | X | | X | X | X | X | X | X | X | | X | X |
| *P. phosphoreum* | | | | | | | | | X | X | X | | X | X | X | | X | X | X | | | X | X | | | X | X | |

N = no[parasite], L = low[parasite], M = medium[parasite], H = high[parasite], C = negative control.
[a] Before inoculation.
[b] After inoculation.

black centres on IAL during cultivation. Although both *Pseudomonas* spp. and *P. phosphoreum* have been documented to suppress the growth of *S. putrefaciens* (Debevere & Boskou, 1996; Gram et al., 2002), it seems unlikely that this and all other $H_2S$-producing bacteria were absent from the current samples.

The spoilage rate and pattern differed between samples with and without parasite homogenate added, however, with opposite effect of what we initially expected. The parasite(+)-samples spoiled similarly to fresh fish from northern temperate waters, whereas the no[parasite] samples showed a quite different and more adverse spoiling manner. Based on these findings, one may hypothesise that the highly hygienic and aseptic mincing process of (*Anisakis*-free) fish fillets, might exclude some important spoilage inhibiting bacteria, thus favouring growth of just a few active spoilers, which again may reduce the shelf-life of the actual products.

## 5. Conclusion

The present storage trial with fish mince added a homogenate of *Anisakis* larvae at three concentrations, showed that bacteria associated with the larvae increased the number of $H_2S$-producing bacteria and the level of TMA/N during storage. The no[parasite] samples, *i.e.* without any parasite additives, spoiled most rapidly and reached the highest PC numbers of bacteria. This again indicates that the parasite microbiota does not necessarily affect the shelf-life adversely, as we suggested in an earlier study (Svanevik et al. 2013). Furthermore, the infection intensity of *Anisakis* larvae did only show a weak negative effect on the spoilage activity. Thus we conclude that the interactions between the microbiota of *Anisakis* larvae and the intrinsic fish microbiota appear to be highly complex and further examinations are needed to describe the ecology of sub-populations during marine fish spoilage.

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