

RESEARCH ARTICLE

Culturable microbiota associated with farmed Atlantic bluefin tuna (*Thunnus thynnus*)

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Abstract – This study examined culturable microbiota associated with farming of Atlantic bluefin tuna (*Thunnus thynnus*), an economically significant aquacultured fish species, in autumn and late spring. While sea water gave higher heterotroph plate counts than tuna skin and gill swabs, the opposite was true for *Vibrio* plate counts. Most bacteria identified from skin and gill swabs at both samplings were Gram-negative and comprised *Pasteurella* and *Moraxella* genera. At both samplings, *Moraxella* was the most abundant genus in internal organs, followed by *Pasteurella* and *Brevundimonas*, which were present in fewer organs. While none of the microbiota identified is known to be pathogenic to tuna, several are spoilage bacteria: *Klebsiella*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Vibrio* and *Weissella*. Total abundance of bacteria was greater on tuna skin than on gills. These results provide information about the bacterial community normally associated with healthy farmed Atlantic bluefin tuna, which will be helpful for assessing how changes in this community may affect farmed tuna health, risk of spoilage and safety of raw and processed tuna.

Keywords: Atlantic bluefin tuna / Adriatic Sea / Culturable bacteria / Pathogens / Spoilage bacteria

1 Introduction

Atlantic bluefin tuna (*Thunnus thynnus*) is a high-value product (Druon et al., 2011), and Croatia is one of the primary Mediterranean producers (Vita and Marin, 2007; Kapetanović et al., 2013a). Aquaculture of this tuna involves capturing juvenile and adult fish from the wild, transferring them into floating cages and fattening them for periods up to 1 year in the case of larger fish, or longer than 1 year in the case of smaller fish (Tičina et al., 2007).

Despite the commercial importance of Atlantic bluefin tuna, little is understood about bacteria naturally associated with the farmed fish (Kapetanović et al., 2006). Various bacteria have been isolated from muscle, kidney, liver and spleen of various fish species (Sousa and Silva-Souza, 2001; Austin, 2002), raising the question of whether the same populations occur in farmed Atlantic bluefin tuna. Since this fish is a highly migratory predator at the top of the trophic web (Ottolenghi, 2008), it takes in food and water abundant in microbiota that may colonise it (Austin, 2002). *Aeromonas*, *Vibrio*, *Photobacterium* and *Mycobacterium* are known to cause disease in tuna (Munday et al., 2003), and the major histamine formers *Morganella morganii*, *Klebsiella pneumo-*

niae and *Hafnia alvei* have been found in frozen tuna (Ben-Gigirey et al., 2002; Fernández-No et al., 2011). *Enterobacter aerogenes*, *Klebsiella planticola* and *Pseudomonas* spp. have been found in spoiled tuna (Ben-Gigirey et al., 2002). The shelf-life of fresh bluefin tuna depends on the initial count of aerobic mesophilic bacteria (Torrieri et al., 2011). Microbiota associated with farmed Atlantic bluefin tuna in the Adriatic have been linked to a disease outbreak caused by *Photobacterium damsela* subsp. *piscicida* (Mladineo et al., 2006).

These observations highlight the need to understand the bacterial community naturally associated with farmed Atlantic bluefin tuna, which may pose a poisoning risk (Ben-Gigirey et al., 1999; Emborg et al., 2005) or an infection risk (Vardić Smrzlić et al., 2012) to tuna and humans (Van Spreekens, 1977; Banja, 2002). Nevertheless, this question has been neglected, with the literature instead focusing on bluefin tuna parasitology and other pathologies (Marino et al., 2003, 2006; Mladineo, 2006; Mladineo and Bočina, 2006; Roberts and Agius, 2008). Some studies have examined health problems of Atlantic bluefin tuna in aquaculture (Munday et al., 2003; Mladineo et al., 2008; Rigos and Katharios, 2010), but they have not correlated these problems to a bacterial community naturally associated with the fish. One study has examined spatiotemporal variability in the bacterial community associated with bluefin tuna larvae (Gatesoupe et al., 2013), but how this

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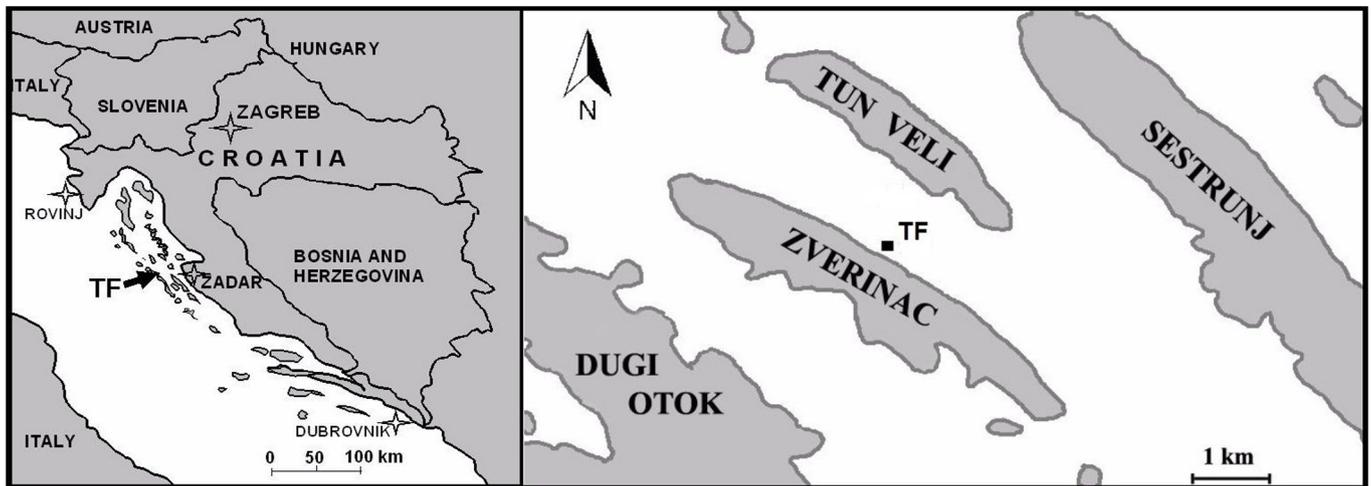


Fig. 1. Sampling locations on a commercial tuna farm (TF) in the Middle Adriatic. (a) Sampling locations within Croatia. (b) Close-up view of sampling locations on the tuna farm.

compares to the community associated with adult fish is unclear.

The present study aimed to examine the microbial community naturally associated with adult Atlantic bluefin tuna in the Adriatic, as a continuation of our earlier work (Kapetanović et al., 2006). In parallel, we examined microbiological quality of the sea water, as an extension of our earlier work evaluating the impact of tuna farming on water quality (Kapetanović et al., 2013a). Skin, gills and internal organs of healthy fish, as well as the rearing sea water, were sampled for bacteria in autumn and late spring. Heterotroph plate counts (HPCs) and *Vibrio* counts were recorded, *Vibrio* counts were correlated with microbiological quality of sea water, and bacterial species were identified in an effort to detect ones dangerous to the tuna.

2 Materials and methods

2.1 Tuna and water sampling

Microbiological analysis was conducted on Atlantic bluefin tuna (*T. thynnus*) on a commercial tuna farm in the Middle Adriatic (N 44°09,896'; E 14°55,779'), as well as on sea water from the same farm (Fig. 1). This farm, like most other farms in Croatia, was established by catching juvenile Atlantic bluefin tuna weighing 8–15 kg (Mišlov Jelavić et al., 2012; Grubišić et al., 2013), transferring them to floating cages and fattening them for at least 18 months (Mišlov Jelavić et al., 2012). Tuna on the study farm were caught by commercial purse-seine fishing vessels in the southern Adriatic Sea during a 1-month period in accord with regulations of the International Commission for Conservation of Atlantic Tunas (www.iccat.int/en). Several captured cohorts were transferred to circular floating growth-out cages with a diameter of 50 m and depth of 20 m, and fed with the following types of fresh fish from the Adriatic Sea: European pilchard (*Sardina pilchardus*), sprat (*Sprattus sprattus*) and European anchovy (*Engraulis encrasicolus*). This diet was supplemented with smaller amounts of defrosted fish from the North Sea, mainly herring (*Clupea harengus*), imported anchovy (*E. encrasicolus*) and Atlantic cod (*Gadus morhua*). One floating cage was

selected for the present study, and only fish inside that cage underwent bacteriological monitoring.

Healthy Atlantic bluefin tuna ($n = 14$) were caught using a baited hook and immediately lifted onto a boat for examination. Skin and gills were swabbed in the autumn of 2007 and 2008, as well as in the spring of 2008 and 2010. Once annually from 2003 to 2010, animals were euthanised, necropsy was performed and internal organs were sampled. Tuna size ranged from 75 to 151 cm, and weight ranged from 6.5 to 39.0 kg (Table 1). Tuna samplings were as follows: one tuna in November 2003; five tuna from June 2004 to November 2005; three tuna in November 2006, June 2007 and November 2007; and five tuna in June and November 2008, June 2009, and June and November 2010.

The water at the fish farm was 55–60 m deep (because of a sloping seabed) and was sampled at depths of 0.5, 3 and 59–60 m using a 6-L Niskin sampler (General Oceanics, Miami, FL, USA). Water was sampled at these depths in the autumn of 2007 and 2008, as well as in the spring of 2008 and 2010, in parallel with swabbing of tuna skin and gills.

2.2 Physicochemical analysis of sea water

Temperature (°C), dissolved oxygen (mg/L) and salinity (‰) were recorded using a multi-probe (UC-12, Kagaku, Tokyo, Japan) and salinometer (Atago, Tokyo, Japan). The oxygen saturation percentage was determined based on a method developed by the United States Geological Survey (2008) as well as based on the measurements of temperature, dissolved oxygen and salinity. At each sampling, each of these parameters was averaged over the three depths to give a mean value for the water column.

2.3 Microbiological analysis of sea water

To determine HPC, samples of sea water were serially diluted with phosphate-buffered saline (PBS, Sigma–Aldrich, St. Louis, MO, USA) and plated in duplicate on Tryptic Soy Agar (BD Difco™, Sparks, MD, USA) supplemented with 1% NaCl (TSANaCl). Plates were incubated at 22 °C. To

Table 1. Total length and weight of Atlantic bluefin tuna (*Thunnus thynnus*) on the study farm, 2003–2010.

| Year | Late spring (n=6) | | | Autumn (n=8) | | |
|-----------|-------------------|-------------------|---------------------------|-------------------|-------------------|---------------------------|
| | Total length (cm) | Total weight (kg) | Time in ranching (months) | Total length (cm) | Total weight (kg) | Time in ranching (months) |
| 2003 | NA | NA | NA | 100.0 | 20.0 | 18 |
| 2004 | 75.0 | 6.5 | 1 | 80.0 | 9.0 | 6 |
| 2005 | 85.0 | 10.0 | 12 | 102 ± 1.4* | 20.3 ± 0.4* | 18 |
| 2006 | NA | NA | NA | 78.0 | 8.5 | 6 |
| 2007 | 97.0 | 12.8 | 12 | 90.0 | 9.0 | 18 |
| 2008 | 82.0 | 8.0 | 1 | 113.0 | 19.0 | 6 |
| 2009 | 93.0 | 12.0 | 12 | NA | NA | NA |
| 2010 | 132.0 | 25.5 | 24 | 151.0 | 39.0 | 30 |
| Mean ± SD | 94.0 ± 20.2 | 12.5 ± 6.8 | | 102 ± 23.1 | 18.1 ± 10.1 | |

NA, not analysed.

determine *Vibrio* count, undiluted and serially diluted sea water samples were plated onto TCBS Agar (BD Difco™) and incubated at 35 °C. This high temperature is useful for detecting *Vibrio* species that can be a risk to public health (Jakšić et al., 2002). Both HPC and *Vibrio* counts were calculated as colony-forming units (CFU) per 1 mL of sea water. At each sampling, HPC and *Vibrio* counts were averaged over the three depths to give a mean value for the water column.

2.4 Swab procedure and analysis

Gills, skin, spleen, liver and kidney were sampled using sterile inoculating loops and cultured on Tryptic Soy Agar (BD Difco™) supplemented with 1% NaCl. Plates were incubated at 22 °C. Simultaneously with loop sampling, gill and skin were also sampled using swab sticks with a cotton apex 1 cm long (Deltalab, Rubi, Spain). Gills and skin below the dorsal fin were swabbed over a 1-cm² area (Kapetanović et al., 2013b). Swab samples were diluted in 10 mL of sterile PBS (Sigma–Aldrich) in tubes, which were stirred and agitated.

Undiluted and 10-fold dilutions of swab samples were then plated onto an appropriate medium to determine HPC and *Vibrio* count. To determine HPC, samples were inoculated onto non-selective TSANaCl, and plates were incubated at 22 °C. To determine *Vibrio* count, samples were inoculated onto selective TCBS Agar (BD Difco™) in duplicate (Moriarty, 1998) and incubated at 35 °C. HPC and *Vibrio* count were calculated as CFU per 1 cm² of gills or skin.

2.5 Bacterial identification

Positive gill and skin swabs from two bluefin tuna sampled in the late spring and two tuna sampled in autumn gave rise to yellow colonies on TCBS Agar. Three single colonies representative of the three observed morphologies were selected and restreaked three times onto fresh TCBS Agar to ensure pure clones. Similarly, representative colonies with different morphologies on positive TSANaCl plates were selected and restreaked three times onto fresh TSANaCl plates.

These colonies were then characterised based on cell morphology, motility, Gram staining, as well as oxidase and

catalase activities (Whitman, 2004). The isolates were characterised phenotypically using the following API kits (bioMérieux, France) according to the manufacturer's instructions: API 20 NE, API 20 E, API Staph, API Coryne and API 50 CHL. Samples were incubated in duplicate at 22 °C (Kent, 1982; Austin and Austin, 1999; Topic Popovic et al., 2007) and at 35 °C. API results were interpreted with the aid of the APIWEB platform (bioMérieux).

The assignment of *Vibrio* strains to the species *V. alginolyticus* was confirmed by partial deoxyribonucleic acid (DNA) sequencing of the *gyrB* gene (Izumi et al., 2007), which is well suited to analysis of bacterial phylogeny because of limited horizontal transmission and presence in all bacterial groups (Luo and Hu, 2008). This gene is also useful for describing *Vibrio* species diversification (Le Roux et al., 2004), potentially even more useful than 16S rRNA sequencing, because the 16S rRNA gene is highly homologous among *Vibrio* spp. (Luo and Hu, 2008). Total DNA was extracted from a single isolate using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) reactions (50 µl) contained 5 µl of DNA template, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 40 pmol of each primer, 1 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA) and DNase/RNase-free water (Sigma–Aldrich). Reaction conditions were as follows: 5 min at 94 °C; 35 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 1 min; and 72 °C for 10 min. PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Foster City, CA, USA) and sequenced using an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) in the DNA Service of the Ruđer Bošković Institute. The sequence was deposited in GenBank under accession number KF407933 and analyzed using BLAST (Altschul et al., 1997).

2.6 Statistical analyses

All statistical analyses were performed using SigmaStat 1.0 (Systat Software, San Jose, CA, USA). Non-parametric statistical analysis was performed to compare the frequencies of different bacteria in late spring and autumn. Differences in the numbers of isolates obtained from skin, gills, or organs (liver, spleen, kidney) across both sampling seasons and

Table 2. Physicochemical characteristics of rearing sea water at sampling locations on the tuna farm.

| | Late spring | | | | Autumn | | | |
|-------------------------|-------------|------|------|------|---------|------|------|------|
| | Average | SD | Min | Max | Average | SD | Min | Max |
| Temperature (°C) | 20.24 | 2.32 | 16.1 | 21.6 | 16.58 | 1.67 | 14.9 | 18.6 |
| Dissolved oxygen (mg/L) | 6.09 | 0.33 | 5.68 | 6.55 | 5.76 | 0.10 | 5.65 | 5.88 |
| Salinity (‰) | 37.65 | 0.26 | 37.3 | 38.1 | 38.4 | 0.09 | 38.3 | 38.5 |

Max, maximum value; Min, minimum value; SD, standard deviation.

Table 3. Heterotroph plate count (HPC) and *Vibrio* count in rearing sea water (log CFU/mL) and on skin and gills of Atlantic bluefin tuna (log CFU/cm²) in two seasons.

| | Spring | | | Autumn | | |
|---|-----------|-----------|-----------|-----------|-----------|-----------|
| | Water | Skin | Gills | Water | Skin | Gills |
| HPC | 4.1 ± 0.3 | 2.2 ± 1.4 | 1.4 ± 0.6 | 3.8 ± 0.2 | 1.9 ± 0.7 | 0.7 ± 0.1 |
| <i>Vibrio</i> (<i>V. alginolyticus</i>) | 0.7 ± 0.8 | 1.6 ± 1.6 | 1.2 ± 1.1 | 0.5 ± 0.6 | 0.9 ± 0.7 | 1.0 ± 0.3 |

Values are average ± standard deviation.

between seasons were assessed for significance using the Mann–Whitney rank sum test. Inter-seasonal differences in HPC and *Vibrio* count based on swab samples were assessed using a *t* test. Differences in HPC and *Vibrio* count on gills, skin and rearing sea water were assessed using the Mann–Whitney test.

3 Results

3.1 Physicochemical properties of sea water

The temperature ($p=0.014$) and dissolved oxygen ($p=0.026$) in the sea water on the Atlantic bluefin tuna farm in our study were significantly higher in late spring than in autumn, whereas salinity ($p=0.001$) was significantly higher in autumn (Mann–Whitney test, $p < 0.05$) (Table 2). All three parameters were combined to determine oxygen saturation, which was lower in autumn (63.86 vs. 69.64%, Mann–Whitney test, $p=0.017$).

3.2 Microbiological analysis of sea water

HPC and *Vibrio* counts tended to be higher in late spring than in autumn (Table 3), although the differences did not achieve statistical significance (Mann–Whitney test, $p > 0.05$).

3.3 HPC and *Vibrio* counts in skin and gill swabs

HPC and *Vibrio* counts were also determined from tuna skin and gills swabbed at the same time that sea water was sampled. As in sea water, both HPC and *Vibrio* count differed between late spring and autumn (Table 3). Within each tissue type (gills and skin), seasonal differences were significant for HPC (Mann–Whitney test, $p < 0.05$). Nevertheless, the seasonal difference was particularly large for HPC on gills, which averaged 1.4 ± 0.6 log CFU/cm² in late spring and 0.7 ± 0.1 log CFU/cm² in autumn (Mann–Whitney test, $p=0.029$). The corresponding seasonal difference for HPC on skin was much smaller (2.2 ± 1.4 vs. 1.9 ± 0.7 log CFU/cm²)

but significant (Mann–Whitney test, $p=0.029$). The corresponding seasonal differences for *Vibrio* count were 1.6 ± 1.6 vs. 0.9 ± 0.7 log CFU/cm² on skin and 1.2 ± 1.1 vs. 1.0 ± 0.3 log CFU/cm² on gills (Mann–Whitney test, $p > 0.05$). These results suggest that HPC in the rearing sea water, together with sea water temperature, was a primary determinant of HPC from skin and gill swabs in our study.

HPC on skin was significantly higher than HPC on gills in late spring and autumn (Mann–Whitney test, $p=0.029$, Table 3). *Vibrio* count was similar between skin and gills within each sampling season, and it was marginally but not significantly higher than in the rearing sea water (Mann–Whitney test, $p > 0.05$).

Phenotypic characterisation of the isolates identified *V. alginolyticus* as the only *Vibrio* species, and this was confirmed by partial DNA sequencing of a representative isolate from skin.

3.4 Microbiological analysis of skin, gills and internal organs

During our study, most microbiota from samples of Atlantic bluefin tuna fluctuated in the same pattern across sampling years, and the predominant microbiota did not vary significantly across years. Therefore, culturable microbiota were analysed in detail from only two sampling seasons. Bacteria were detected in 70% of the total set of skin, gill and organ swab samples, and the proportion of positive samples was similar between late spring and autumn for skin, gills, liver and spleen (Mann–Whitney test, $p > 0.05$). In contrast, the number of colonies obtained from kidney was significantly higher in autumn than in spring (Mann–Whitney test, $p=0.005$).

3.4.1 Analysis of isolates from skin and gills

A total of 433 bacterial isolates on TSANaCl plates were identified from skin swabs (Table 4). Of the 132 isolates from skin in the spring sampling, 47.0% were *Staphylococcus lentus*

Table 4. Bacteria identified on skin and gill swabs of farmed Atlantic bluefin tuna in spring and autumn.

| | Spring | | | | Autumn | | | |
|--|--------|------|-------|------|--------|------|-------|------|
| | Skin | | Gills | | Skin | | Gills | |
| | No. | % | No. | % | No. | % | No. | % |
| <i>Brevundimonas vesicularis</i> | | | | | | | 8 | 15.4 |
| <i>Corynebacterium propinquum</i> | | | | | 12 | 7.3 | | |
| <i>Moraxella</i> spp. | 35 | 26.5 | 25 | 29.4 | 17 | 10.4 | 19 | 36.5 |
| <i>Pasteurella</i> spp. | 1 | 0.7 | 60 | 70.6 | 84 | 51.2 | 15 | 28.9 |
| <i>Pseudomonas</i> spp. | | | | | 2 | 1.2 | | |
| <i>Staphylococcus lentus</i> | 62 | 47.0 | | | | | | |
| <i>Staphylococcus xyloso</i> | 17 | 12.9 | | | 13 | 7.9 | 2 | 3.8 |
| <i>Staphylococcus</i> spp. | 5 | 3.8 | | | 36 | 22.0 | 7 | 13.5 |
| <i>Weeksella virosa</i> / <i>Empedobacter brevis</i> | 12 | 9.1 | | | | | 1 | 1.9 |

No., number of isolates; %, relative frequency among all isolates.

and 26.5% were *Moraxella* spp. Present at lower frequencies were *S. xyloso*, *Weeksella virosa*/*Empedobacter brevis*, *Staphylococcus* spp. and *Pasteurella* spp. This distribution changed in the autumn: of the 164 isolates from skin, 51.2% were *Pasteurella* spp. and 22.0% were *Staphylococcus* spp. Present at lower frequencies were *Moraxella* spp., *S. xyloso*, *Corynebacterium propinquum* and *Pseudomonas* spp. Combining the data from both samplings indicates that the dominant bacteria on the skin were *Pasteurella* spp., *Moraxella* spp., *Staphylococcus* spp., and *S. xyloso*; these species accounted for 70% of the total bacterial community on the skin.

A total of 137 bacterial isolates were identified from gill swabs, comprising 85 isolates identified in the spring and 52 in the autumn (Table 4). Of the isolates in the spring, 70.6% were *Pasteurella* spp., while 29.4% were *Moraxella* spp. These bacteria also predominated in the autumn, when their respective frequencies were 36.5% and 28.9%. Present at lower frequencies were *Brevundimonas vesicularis* (15.4%), *Staphylococcus* spp. (13.5%) and *S. xyloso* (3.8%). These results indicate that the most abundant bacteria on gills were *Pasteurella* spp. and *Moraxella* spp., accounting for 86% of the total bacterial community on gills.

3.4.2 Analysis of isolates from internal organs

A total of 166 isolates on TSANaCl plates were identified from sampling of tuna internal organs (Table 5), 24 in the late spring and 112 in the autumn (Mann–Whitney test, $p = 0.001$). The isolates comprised 11 bacterial genera and 14 species, of which 126 were Gram-negative and 10 were Gram-positive. Most isolates came from the kidney (90), followed by the liver (53) and finally the spleen (23).

Liver and spleen contained the smallest number of bacterial isolates, and total bacterial abundance was similar in late spring and autumn (Table 5). Kidney contained the largest number of species (7), and this number was significantly higher in autumn than in late spring (Mann–Whitney test, $p = 0.012$).

Among identified isolates, *Moraxella* spp. predominated: it accounted for 33.7% of all isolates, and it was isolated from all three internal organs at both samplings. The next most frequent

bacteria were *Pasteurella* spp. (19.9%) followed by *B. vesicularis* (13.2%). These bacteria were isolated at both samplings, but not in all three internal organs.

S. lentus was isolated only in late spring, while *B. diminuta*/*Oligella urethralis*, *Klebsiella* spp., *Pseudomonas* spp. and *Weeksella virosa*/*Empedobacter brevis* were isolated only in autumn. Bacteria were excluded from the analysis if fewer than 4CFU were isolated.

No significant seasonal differences were observed in the frequencies of *Pasteurella* spp., *Moraxella* spp., or *B. vesicularis* ($p > 0.05$). In contrast, HPC was significantly higher in the late spring than in the autumn (Mann–Whitney test, $p = 0.029$, Table 3).

4 Discussion

Although the Atlanta bluefin tuna in our study were clinically healthy, we isolated a substantial number of bacteria from their tissues, suggesting that farmed tuna are associated with a bacterial community, similar to studies in other healthy fish (Sousa and Silva-Souza, 2001; Austin, 2002). Our results on the normal bacterial community associated with Atlantic bluefin tuna during rearing may help in the detection and characterisation of changes in this community that may benefit or harm tuna, such as following environmental disturbances or immunocompromise.

In our study, HPC was much lower on skin and gills than in rearing sea water. This may reflect stimulation of bacterial growth in sea water as the temperature rises in late spring. In contrast to HPC, *Vibrio* count was higher on skin and gills than in sea water at both samplings. This suggests that sea water influences the bacterial community on the skin (Kapetanović et al., 2013a; Valdenegro-Vega et al., 2013), and that this influence is likely to be complex. Such influence may help explain why Gram-positive bacteria were so abundant on the tuna skin in our study. Future research should clarify whether the microbiota on skin and gills comes from the sea water, and verify whether the microbiota is specifically colonising gill or skin. Such research should also examine to what extent the variations in bacterial community composition in our study were due to temperature or other

Table 5. Bacteria identified on liver, spleen and kidney of farmed Atlantic bluefin tuna in spring and autumn.

| | Spring | | | | | | Autumn | | | | | |
|--|--------|------|--------|------|--------|------|--------|------|--------|------|--------|------|
| | Liver | | Spleen | | Kidney | | Liver | | Spleen | | Kidney | |
| | No. | % |
| <i>Brevundimonas diminuta/Oligella uretralis</i> | | | | | | | 5 | 11.1 | | | | |
| <i>Brevundimonas vesicularis</i> | | | | | 1 | 14.2 | | | 4 | 28.6 | 17 | 20.5 |
| <i>Corynebacterium propinquum</i> | | | | | | | | | | | 3 | 3.6 |
| <i>Klebsiella</i> spp. | | | | | | | | | | | 4 | 4.8 |
| <i>Moraxella lacunata</i> | | | | | 2 | 28.6 | | | | | | |
| <i>Moraxella</i> spp. | 6 | 75.0 | 3 | 33.3 | 2 | 28.6 | 11 | 24.4 | 10 | 71.4 | 24 | 28.9 |
| <i>Pasteurella multocida</i> | | | 1 | 11.1 | | | | | | | | |
| <i>Pasteurella</i> spp. | | | 2 | 22.2 | 2 | 28.6 | 29 | 64.4 | | | | |
| <i>Pseudomonas</i> spp. | | | | | | | | | | | 5 | 6.0 |
| <i>Staphylococcus lentus</i> | 2 | 25.0 | 3 | 33.3 | | | | | | | | |
| <i>Weissella viridescens</i> | | | | | | | | | | | 2 | 2.4 |
| <i>Weeksella virosa/Empedobacter brevis</i> | | | | | | | | | | | 28 | 33.7 |

No., number of isolates; %, relative frequency among all isolates.

seasonal changes, or perhaps to other parameters such as feed, water quality, and time in ranching.

Our results are in agreement with Valdenegro-Vega et al. (2013), who reported obtaining more isolates of culturable bacteria from the gills of southern bluefin tuna than from internal organs. Less clear is why the internal organs in our and their study showed the presence of bacteria. This raises the possibility that the fish in our study were in early stages of infection (Austin, 2002). Consistent with this idea, *Moraxella* was abundant in skin, gills and internal organs in our fish, and this genus has also been found in internal organs of Mediterranean fish on farms showing slow growth, lower production efficiency, and poor feed conversion (Addis et al., 2010). The maximum HPC on skin was $2.2 \pm 1.4 \log \text{CFU/cm}^2$, which is half the minimum HPC on the skin of sardines (*S. pilchardus*) from the Adriatic Sea ($4.08 \log \text{CFU/cm}^2$) (Gennari et al., 1999). It is also below the threshold of 10^7CFU/g at which spoilage begins (Laksmanan, 2000). In addition, none of the bacteria that we isolated from internal organs has ever been associated with disease outbreaks in Atlantic bluefin tuna from the Adriatic Sea (Mladineo et al., 2006, 2008) or elsewhere in the world (Munday et al., 2003). In fact, some of the bacteria identified in our samples are similar to the genera that occur in the bacterial communities associated with apparently healthy European sea bass (*Dicentrarchus labrax*) in the Adriatic Sea. These genera are Gram-negative (primarily *Moraxella*, *Pasteurella*, Non-fermenters, *Ochrobactrum*) as well as Gram-positive (*Staphylococcus*, *Micrococcus*, *Corynebacterium*) (Kapetanović et al., 2011).

The bacterial community associated with tuna in our study comprised at least 8–9 genera, most of which were Gram-negative. Similarly, most microbiota in Adriatic sardines were found to be Gram-negative (Gennari et al., 1999). The proportion of isolates that was Gram-positive in our study was significantly higher on the skin (49.0%) than on the gills (6.6%), and this proportion differed among the liver (8.7%), spleen (5.7%) and kidney (5.6%).

Absence of mortalities in our study may be attributed to the absence of pathogenic *Vibrio* and *Photobacterium* (Munday

et al., 2003; Valdenegro-Vega et al., 2013). Similarly, Valdenegro-Vega et al. (2013) failed to observe a relationship between isolated bacteria and moribund or dead fish. These present and previous results suggest that these culturable bacteria do not influence the health of Atlantic bluefin tuna, at least under certain environmental conditions.

Several bacteria that we identified as part of the normal community associated with farmed tuna can cause spoilage of fresh tuna meat and tuna products. *Pseudomonas*, *Moraxella* and *Vibrio* are known as fish spoilage bacteria (Banja, 2002), and *Pseudomonas* spp. has been isolated from spoiled tuna meat (Ben-Gigirey et al., 2002). *V. alginolyticus* is a weak histamine former (Björnsdóttir-Butler et al., 2010), *S. xylosum* is a histamine former in salted semi-preserved anchovies (Fernández-No et al., 2011), and *Klebsiella* is a major histamine former in frozen tuna (Taylor and Speckhard, 1983; Ben-Gigirey et al., 2002). In contrast to previous reports in which *Klebsiella* was isolated from tuna skin but not muscle (Ben-Gigirey et al., 2002), we isolated it from tuna kidney. From this organ we also isolated *Weissella*, which has been linked to meat spoilage (Borch et al., 1996). While most spoilage bacteria in our tuna were less abundant at one of the samplings, suggesting that their levels are not always high, their presence during processing and storage may affect the characteristics and quality of fresh tuna meat and tuna products.

Our results should be verified and extended in further studies involving a larger number of samplings, as well as different isolation techniques and incubation media and temperatures, all of which can bias the results. In addition, we pooled very small samples across sampling years, which seemed justified given the lack of microbiota variation during the study period. However, more extensive sampling is needed to ensure that true variation was not missed.

5 Conclusions

We characterised bacteria that are naturally associated with healthy farmed Atlantic bluefin tuna. These results will provide

a valuable baseline reference for future studies of how farming conditions affect the health of farmed tuna, risk of food spoilage and the safety of raw and processed tuna as food for humans. To this end, future work should analyse the seasonal dynamics of bacterial abundance and diversity in farmed tuna.

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