Differences in the gut microbiota between wild and domestic *Acipenser ruthenus* evaluated by Denaturing Gradient Gel Electrophoresis

Received for publication, September 15, 2012
Accepted, January 15, 2013

GIANINA M. BACANU, LUCIAN OPREA
„Dunărea de Jos” University of Galați, Faculty of Food Science and Engineering, 47, Domneasca str., 800008-Galati, Romania,
E-mail: gianinabacanu@yahoo.com

Abstract

The intestinal microbiota of fish is composed of a collection of aerobic, facultative anaerobic and obligate anaerobic bacteria that are assumed to play an important role in digestion and disease control. Fish intestinal microbiota changes with a lot of factors like water quality, culture systems, species, age, diet etc. The present study focuses on microbial diversity in the intestinal tract of the sterlet (*Acipenser ruthenus*, Linnaeus, 1758). The fish were collected from Lower Danube River (wild) and from an Industrial Flow-Through Aquaculture System - FTAS (domestic). Intestinal content was characterized by molecular methods. Genomic DNA was extracted from each fish gut and polymerase chain reaction (PCR) was used to amplify the conserved 16S ribosomal RNA gene. Denaturing Gradient Gel Electrophoresis (DGGE) of PCR amplified 16S rDNA were used to analyze the microbial community diversity and community organization in the gut. The results showed different microbial profiles from domestic and wild sterlet. PCR-DGGE profile revealed high diversity of microbiota in domestic fish.

Key words: fish sterlet, gut microbiota, PCR-DGGE

Introduction

In recent years, sturgeon culture has developed into a very successful industry, due to the precious black caviar and meat (Geraylou Z. et al [14]). Sturgeons is one of the oldest anadromous (potamodromous) group among the bony fishes with 27 species (Bahmani et al. [6]; Asadi et al. [2]), including six genera: *Acipenser*, *Huso*, *Scaphirhynchus*, *Pseudoscaphirhynchus*, Polyodon, Psephurus (Bemis et al. [7]; Askarian et al. [4]). Of the six species, living in the Danube River, four of them (beluga-*Huso huso*, Russian/Danube sturgeon-*Acipenser gueldenstaedtii*, stellate sturgeon-*Acipenser stellatus* and ship/bastard sturgeon-*Acipenser nudiventris*) are critically endangered (www.iucnredlist.org).

The sterlet (*Acipenser ruthenus*) is vulnerable and Atlantic/European sturgeon (*Acipenser sturio*), critically endangered in the world, is very rare or perhaps disappeared from Danube. Every year the stocks decrease because of over fishing, loss of habitat and decrease of water quality.

One of the most important issues in microbial ecology of the gastrointestinal tract is the understanding of how important factors (systems, water, feed, species and age) influence gut microbiota. The gut microbiota usually refers to a very complex and dynamic microbial ecosystem that colonizes the gastrointestinal tract of an animal (Sukanta K. Nayak [27]). The mammalian gastrointestinal tract contains an enormous variety of aerobic and anaerobic microbes that interact in its complex ecosystem (Nicholson et al [22]), but that of fish is believed to be simpler and less in number than that of endothermic animals (Trust et al. [31]; Sakata [26]).
It is known that the microbiota of fish contribute to important key functions, such as development, immunity and xenobic metabolism (Bates et al. [5]; Navarrete et al. [21]). The microbial community has an important role in the health and nutrition of the host (Burr, et al [8]; Sakata [26]; Ringo et al. [25]; Thompson et al. [30]; Verschuere et al. [32]; Suzer et al. [28]). Evidence for the role of microbiota in fish was recently revealed (Bates J. et al [5]; P. Navarrete et al [21]). For a very long time, it was believed that the activity of intestinal microbiota in the host is correlated with longevity of host (Metchnikoff, [18]). Microbiota may serve as a supplementary source of food, and microbial activity in the digestive tract may be a source of vitamins, essential amino acids and fatty acids (Dall & Moriarty [9]; Sakata [26]). It is important to study microbial diversity not only for basic scientific research, but also to understand the link between diversity and community structure and function (Kirk, J. L. et al [17]). Some information is available on the microbiota on external surface (skin, gills and fins) and in the gastrointestinal (GI) tract of sturgeon (Shenavar Masouleh et al. [29]; Delaedt et al. [12]; Huys et al. [16]; Akrami et al. [3]; Askarian et al. [4]; Ghanbari et al. [15]).

The present research was undertaken to characterise the microbial communities profile of fish gut (domestic and wild sterlet) with a combination of molecular fingerprinting. This research can give better view of the microbial differences from wild and domestic fish of some species. Askarian F. [4] in his study, suggested to use DGGE to evaluate the bacterial gut community in sturgeon and also Geraylou Z. [14] mentions there are not has been studied yet sturgeon species in particular. Only one preliminary study of Siberian sturgeon (Acipenser baerii) has investigated the gut microbiota by DGGE (Delaedt et al. [12]).

Denaturing gradient gel electrophoresis (DGGE) has become a widely used tool to examine microbial diversity and community structure, but no systematic comparison has been made of the DGGE profiles obtained when different hypervariable (V) regions are amplified from the same community DNA samples (Zhongtang et al. [35]).

To detect and quantify these small differences, the power of statistical tools, such as clustering analysis, principal component analysis (PCA) were evaluated.

The best our knowledge, this is the first study tracking the bacterial communities, through culture – independent methods, from intestinal of wild and domestic sterlet.

Material and Methods

Microbial communities were studied on two groups of sterlet: one from Danube River (wild) and one from Industrial Flow-Through Aquaculture System farm (domestic). The wild fish have been caught in January 2012 out of area Chiscani-Gropeni, Braila county and the domestic fish were arrived from Horia farm, Tulcea county, in the same period, also (Figure 1). Immediately after the catch all fish were measured and weighed (Table 1 and Table 2).

![Fig. 1. The area were fish have been grow (D) and were been fishing (W)](image-url)
Differences in the gut microbiota between wild and domestic Acipenser ruthenus evaluated by Denaturing Gradient Gel Electrophoresis

Table 1. Length and weight of wild fish samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Fishing area</th>
<th>Length (cm)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>Chiscani Gropeni</td>
<td>42</td>
<td>310</td>
</tr>
<tr>
<td>W2</td>
<td>Chiscani Gropeni</td>
<td>44</td>
<td>360</td>
</tr>
<tr>
<td>W3</td>
<td>Chiscani Gropeni</td>
<td>38</td>
<td>300</td>
</tr>
<tr>
<td>W4</td>
<td>Chiscani Gropeni</td>
<td>45</td>
<td>390</td>
</tr>
<tr>
<td>W5</td>
<td>Chiscani Gropeni</td>
<td>48</td>
<td>350</td>
</tr>
<tr>
<td>W6</td>
<td>Chiscani Gropeni</td>
<td>43</td>
<td>340</td>
</tr>
</tbody>
</table>

Table 2. Length and weight of domestic fish samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Experimental variant/tank</th>
<th>Length (cm)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>V1</td>
<td>30,7</td>
<td>98</td>
</tr>
<tr>
<td>D2</td>
<td>V1</td>
<td>25,7</td>
<td>58</td>
</tr>
<tr>
<td>D3</td>
<td>V1</td>
<td>32,5</td>
<td>117</td>
</tr>
<tr>
<td>D4</td>
<td>V2</td>
<td>25,5</td>
<td>46</td>
</tr>
<tr>
<td>D5</td>
<td>V2</td>
<td>24,5</td>
<td>52</td>
</tr>
<tr>
<td>D6</td>
<td>V2</td>
<td>23</td>
<td>44</td>
</tr>
</tbody>
</table>

For domestic fish, the experiments were conducted during five months (October 2011-March 2012), in an industrial flow-through aquaculture system. The system was formed by four breeding units, type circular tanks, with a volume of 300 liters. The water comes from 80 m deep drilled well. As biological material, five months white sterlet juveniles has been used, with an average body weight of 15 g/fish, imported from Germany. Two variants were experimented: V1 and V2, with the same stocking density, i.e. 31 fish/unit. During the experiment, the same type of extruded pellets of 2 mm granulation and different crude protein content (50% in V1 and 54% in V2) was distributed in all units with the same feeding level of 5 g/kg metabolic weight (1,2% of total biomass). The fish biomass in each tank was determined biweekly and the daily ration adjusted accordingly. It is noted that during the experiment, temperature ranged within 14°C to 15°C.

Microbial communities was investigated in the hindgut (intestine with spiral valve) of sturgeon fish. Twelve fish (six for each group) were disinfected with alcohol (70%) before opening, than were dissected and the content of the spiral valve was gently removed under sterile conditions. Gut contents were separately collected in sterile Eppendorf tubes under a laminar flow. The samples were frozen at -80°C until analysis.

**Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)**

Total genomic DNA of gut contents was extracted using the DNA Stool Mini-Kit (Qiagen) according with the manufacture’s protocol (Mei Li et al. [20]). All DNA were analyzed by 1% agarose gel electrophoresis for 30 minutes at 100V. DNA extracted from a microbial population can be used to identify the genetic diversity of the dominant populations by PCR and DGGE (Muyzer et al., [21]).

Are used the specific bacterial universal primer sets 27f (GTTTGATCCTGGCTCAG) and 1369r (GCCCGGGAAACGTATTCCACCG) for the first PCR, 968gc(CGCCCCG GGCACGCCCCGCGCCGGGCGGGCAGGGGGAACGCAGAAACTC) and 1369r (GCCCGGGAAACGTATTCCACCG) for the second PCR to amplified the genomic DNA. The PCRs were performed as follows: genomic DNA was denatured at 98°C for 1 min...
and amplified by 20 cycles of 98°C of the first PCR contained the template, 1x Reaction Buffer, 200µM of dNTPs and 1 µl of Phire Hot Start II DNA polymerase (Finnzymes). The second PCR was performed using 1 µl of the first PCR product in a 50 µl reaction mixture containing the same reagents as the first PCR. The conditions of the second PCR were identical to the first reaction. The first and final PCR product was analyzed on a 1% agarose gel for 30 minutes at 100V and visualized by UV illumination after SYBRSafe (Invitrogen) staining to evaluate the size of the amplified fragments (Zhongtang et al. [35]).

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified V6-V8 regions of the bacterial 16S rRNA gene was used to obtain a molecular fingerprint of the microbial communities in all samples. Briefly, assays were performed in a BioRad© DCODE Universal Mutation Detection System™. Analysis of the amplicons was performed on 8% polyacrylamide gels containing a urea-formamide gradient from 30% to 60% (a 100% urea-formamide solution consists of 7 M urea and 40% [vol/vol] formamide). PCR samples were applied to gels in aliquots of 10 µl per lane. Electrophoresis was performed in 0.5% Tris-acetate-EDTA buffer (pH 8.0) at 60°C and 85 V for 16 h. The gels were silver stained and air dried, after which we were examined (Zoetendal E.G. [34]).

The DGGE bands were detected using the band-searching algorithm of BioNumerics software. After normalization of the gels, only those bands with a peak height intensity exceeding 2.0% of the strongest band in each lane were included in further analyses. The calculation of the similarities is based on the Pearson product-moment correlation coefficient and results in a distance matrix (Pearson, K. [23]). Unweighted pair group method using arithmetic average (UPGMA) was performed, and corresponding dendrograms showing the relationships between the DGGE profiles were constructed. Clustering analysis, PCA, were performed with Bionumerics 3.0. By using PCA analysis, the different data of the complex DGGE patterns of one sample could be reduced to one point in a three dimensional space. For PCA, fingerprint types can only be processed by generating a band-matching table first. All bands are divided into classes of common bands and for each pattern, a particular band class can have two states: present or absent (binary matrix).

Results and Discussion

A series of exogenous and endogenous factors can affect the establishment and nature of microbial composition in the gastrointestinal tract of fish. The developmental stage of fish, gut structure, the surrounding environment like ambient water temperature, rearing and farming conditions, different types of chemical, antibiotics, pesticides, herbicides, insecticides, feed and feeding conditions are very critical factors that affect the initial colonization and the subsequent establishment process (Sukanta, [27]).

Denaturing gradient gel electrophoresis (DGGE) finger prints is a useful tool for comparing complex microbial community profiles from different environmental samples (D.Merrifield et al [10]; Dilly et al [13]). DGGE is undeniably a valuable approach in screening complex ecosystems on a large scale and in analyzing various environmental samples in a reduced amount of time (Diarrassouba, [11]). The use of DGGE with 16S rRNA gene fragments, regenerated with group-specific primers, was evaluated by comparing the specific DGGE patterns of activated gut samples of same species of fish from different types of area.

Total DNA was extracted, the V6-V8 region of 16SrRNA genes was amplified and the resulting PCR fragments were separated by DGGE. All the PCR products were of the expected length, approximately 200 bp (Figure 2 and Figure 3).
Differences in the gut microbiota between wild and domestic *Acipenser ruthenus* evaluated by Denaturing Gradient Gel Electrophoresis

**Fig. 2.** Agarose gel (1 %) showing the PCR products for all domestic fish. Lane M: DNA 1Kb ladder. The numbers (D1-D6) indicate biological replicates.

**Fig. 3.** Agarose gel (1 %) showing the PCR products for all wild fish. Lane M: DNA 1Kb ladder. The numbers (W1-W6) indicate biological replicates.

After the second PCR round, was observed visible differences on PCR product concentration, based on band intensities for domestic fish. For gut samples D4, D5, D6 (correspond to variant experimental V2) intensity of bands are more evident than D1, D2, D3 (correspond to variant experimental V1). For wild fish the intensity of PCR bands are more close, only one exception for samples W2 were the intensity is very low.

After the PCR tests the samples were loaded on a DGGE gel. Denaturing gradient gel electrophoresis analysis showed the microbial communities present in all gut samples. On a DGGE gel communities can be analyzed in a rapid way and each band should correspond with only one bacterial species. The results of the DGGE gel can be found in Figure 4.

**Fig. 4.** Denaturing gradient gel electrophoresis profiles. UPGMA dendrogram showing similarity of DGGE profiles of the gut fish of the domestic and wild sterlet. Dendrogram was determined by calculating similarity indices of the densiometric curves of the profiles compared by using the Pearson product-moment correlation. (D1-D6) indicate biological replicates of domestic fish and (W1-W6) indicate biological replicates of wild fish. M-marker.

In our case the density of number of DGGE band is correlate with the intensity of the PCR product. Looking at the visible bands on the gels, there are big differences between the
GIANINA M. BACANU, LUCIAN OPREA

Gut samples from domestic sterlet and wild sterlet. This indicates that the microflora investigated differ on wild and domestic. DGGE profiles of gut samples showed a high number of bands for domestic fish. Also the relative abundance of the DGGE bands revealed that the intestinal community composition clustered significantly according to area type. Gut samples of the fish from FTAS farm (domestic) were clearly separated from the fish from river (wild). Although gut microbiota of each group clustered apart from one another, intergroup variation of intestinal bacteria in each treatment group from FTAS farm was also observed (Figure 4). This intergroup variation was also more pronounced. In general, the gut microbiota of domestic fish group can be characterized by the presence a high abundance of bands. In the cluster analysis, we can see how the profiles clustered in function of the experimental variant (V1 or V2). The microbial diversity was characterised by a very high similarity between fish of some variant (tank). The profile obtained by DGGE with the samples collected from the river appeared less variable than those from system. A more detailed examination of the gels using image analysis software, allowed us to confirm an apparent common pattern in the dynamics of the gut microbial communities in the two tanks. The principal component analyses (PCA) show the same things, there are two distinct groups, one domestic and one wild sterlet. Also, with the PCA analyses we can see for the domestic sterlet intergroup variations, there are group by experimental variant, its means also the fed have impact of gut microbiota (Figure 5).

The temperature for the domestic sterlet was constant between 14-15°C and for the wild sterlet was around 4-5°C and also the feed was different. High temperature increase number of bacteria species, for domestic sterlet, in our case. Than the principal explanation, in our study, could be that the environmental conditions and feed can have a big impact on gut microbiota.

![Fig. 5. Principal components analysis with the PCR-DGGE profiles. Distribution of samples (W1–W6 and D1–D6)](image)

The dominant bands that seem to be present in many samples, and specially 2 bands (indicated with an white bow in the DGGE gel, Figure 4). If this can be confirmed by sequencing data, we could conclude that a lot of factors (culture system, tanks, feed, temperature, water quality etc) do not totally shape the microbial communities in the gut of domestic sterlet and wild sterlet. At least one dominant population seems to be present in all fish. The sequence data is needed to confirm the effect of environmental conditions in the microbial colonization of fish gut.
Conclusions

We analyzed the gut bacterial composition of sterlet using molecular methods. The application of molecular tools to intestinal microbiology has greatly facilitated the study of the complex microbial community.

A not very complex profile was observed in the gut of fish. DGGE analysis of the bacterial DNA, isolated from gut, indicated that the bacterial profile is different between wild and domestic sterlet, but also they have some common bacterial populations. According to a PCA analysis of DGGE patterns the microbial composition in sterlet gut, as variant experimental and area locations were clustered in different distinct groups. This observed enhancement of microbiota composition is related, first of all, to environmental conditions.

Acknowledgements

Researches was conducted in the framework of the project POSDRU-89/1.5/S/52432-Organizing the national interest postdoctoral school of „Applied biotechnologies” with impact on Romanian bioeconomy project co-financed by the European Social Fund through the Sectoral Operational Programme Human Resources Development 2007-2013.

References


