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New insights on the macromolecular building of rainbow trout (*O. mykiss*) intestine: FTIR Imaging and histological correlative study

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ABSTRACT

Fish intestine plays various physiological functions and its integrity is essential to guarantee fish growth and welfare. Several laboratory techniques have been proposed for studying morpho-physiology of fish intestine, but classical histology still represents one of the gold standards. Nevertheless, this technique is time consuming and does not provide comprehensive information on the biochemical composition of tissues. The present study, introduces a new approach for studying fish intestine, the Fourier Transform Infrared (FTIR) spectroscopy. This is a fast, label-free analytical technique, which analyses the vibrational transitions induced on tissues by the interaction with the electromagnetic radiation. On the base of vibrational features of some functional groups classes and molecular bonds, FTIR allows the analysis of the macromolecular composition of the sample. Rainbow trout (*O. mykiss*) represents an important species for modern aquaculture and deeper knowledge about its intestine anatomy and macromolecular composition is desirable. In the present study, besides the traditional histological analysis, the biochemical building of the intestine mucosa was analyzed for the first time by FTIR. Spectral data can be considered a complement to traditional histological analysis.

1. Introduction

The gastrointestinal tract of vertebrates along with its single layer of epithelial cells has various biological functions. It constitutes the largest and most important barrier to the external environment (Groschwitz and Hogan, 2009), maintaining an effective defence against pathogens and tolerance toward dietary antigens (Peterson and Artis, 2014). At the same time, intestinal epithelium acts as a selective permeable barrier for dietary nutrients, electrolytes and water and plays a fundamental role in the absorption of nutrients, osmotic balance, recycling of enzymes and macronutrients (Rombout et al., 1985; Álvarez-Pellitero, 2011; Urán et al., 2008). Epithelial cells are also crucial mediators of mucosal innate and adaptive immunity, important for distinguishing pathogens from commensal microbiota living in the gut (Kinnebrew and Pamer, 2012; Donaldson et al., 2016).

In fish species, the integrity and control of the intestinal barrier is often attenuated by both nutritional and immunological challenges. Therefore, intestine integrity is nowadays assumed as a key factor for growth and welfare of farmed fish. At this regard, many traditional analytical techniques and methods are available for gut analysis, including visual inspection and microscopy analysis (Randazzo et al., 2015; Silva et al., 2017), as well as biochemical (Baker et al., 2014), molecular and proteome assays (Wulff et al., 2012). All these techniques and methods play a pivotal role in the analysis of fish intestine and some of them are used as gold standards and regulation methods serving scientific researches due to their relative validity and accuracy. Nevertheless, they are normally expensive, time-consuming and laborious.

Salmonids play a key role in the global aquaculture production (ICES, 2006) and their intestine has been studied in several works due to its central role in nutrient absorption and immune response (Urán et al., 2008; Martin et al., 2012; Sahlmann et al., 2013). Furthermore, a detailed histological analysis of the intestine of Atlantic salmon has recently been performed (Løkka et al., 2013). Despite the great importance of trout for aquaculture, studies on the intestinal anatomy are old (Burnstock, 1959; Weinreb and Bilstad, 1955). The more recent studies on the intestine of this species were mainly performed through histological (Baeverfjord and Krogdahl, 1996; Ringø et al., 2007;

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Krogdahl et al., 2010) and, in some cases, transcriptomic approaches (Martin et al., 2016).

Currently, there is a growing interest in the application of the Fourier Transform Infrared (FTIR) spectroscopy to the biochemical field. This is a fast, label-free analytical technique, which analyses the vibrational transitions induced on matter by the interaction with the electromagnetic radiation in an appropriate continuous range of frequencies. It couples a quick response in terms of biochemical composition of the sample to low cost-effectiveness and short time of sample preparation and data acquisition. The mid-infrared region, which cover the spectral range from 4000 cm^{-1} to 400 cm^{-1}, is widely used for investigating the fundamental vibrational modes, mainly stretching and bending, of chemical bonds (Stuart, 2004). The coupling of IR spectrometers with visible microscopes led to the development of FTIR Imaging spectroscopy which allow performing the infrared imaging analysis of non-homogeneous biological samples, such as tissues and cells. Small modifications in the position and absorption of IR peaks can be correlated to changes in the biochemical composition and metabolic processes of biological samples, ascribable to specific benign and malignant pathologies, as well as to the presence of environmental contaminant agents or various cellular differentiation steps (Giorgini et al., 2011; Giorgini et al., 2015a; Giorgini et al., 2015b; Lipiec et al., 2013; Mihoubi et al., 2017; Sreedhar et al., 2015; Tosi et al., 2011). FTIR Imaging spectroscopy requires thin sections of samples (5–10 μm thick tissues from surgical resection or monolayer cell cultures) on which the IR maps can be acquired on areas previously selected by the microscope. The spatial resolution is limited only by diffraction limits and this allows researchers to characterize specific subcellular details, giving the possibility to mutually relate the vibrational local features with the morphology of the different compartments of the sample (Baker et al., 2014; Petibois et al., 2007). Owing to the large number of spectral data collected in a single map, specific software has been developed to highlight similarities and differences in a simple readout format by performing multivariate statistical analysis (Lipiec et al., 2013; Walsh et al., 2009).

Recently, our group has successfully applied this analytical technique to investigate various issues concerning fish species, such as the macromolecular characterization of zebrafish oocytes (Carnevali et al., 2009; Giorgini et al., 2010; Gioacchini et al., 2012) and seabream liver (Carnevali et al., 2017).

Based on these encouraging results, in the present, for the first time, FTIR Imaging spectroscopy was applied to analyse samples of rainbow trout (*O. mykiss*) medium and hind intestinal tracts, with emphasis on the mucosa macromolecular composition. A comparison of the FTIR results with more traditional histological analysis (haematoxylin and eosin as a conventional morphological staining, trichrome stains with aniline blue for connective tissues and Periodic acid–Schiff (PAS) for mucins) was also performed. The spectral data were successfully correlated to the histological ones, providing a deeper insight into the macromolecular composition of intestinal mucosa of rainbow trout.

2. Materials and methods

2.1. Ethics

All procedures involving animals were conducted in line with Italian legislation on experimental animals. Optimal rearing conditions were applied throughout the study, and all efforts were made to minimize animal suffering by using an anesthetic (MS222; Sigma Aldrich).

2.2. Fish and sampling

Rainbow trout (*O. mykiss*) were reared at the University of Udine animal's facility. Five specimens were euthanized using a lethal dose of MS222 (1 g L^{-1}). Then, intestine was quickly dissected from each fish and portions of medium intestine (MI, corresponding to the tract immediately behind pyloric caeca), and hind intestine (HI, corresponding to the distal portion of intestine, excluding the rectum) (Fig. 1) were immediately fixed in Bouin solution (Sigma Aldrich) for histological analysis or frozen at −80 °C for FTIR Imaging analysis.

Fig. 1. Schematic representation of rainbow trout (*O. mikyss*) gastrointestinal tract: S, stomac; pc, pyloric caeca; MI, medium intestine; HI, hind intestine, and R, rectum.

2.3. Histological analysis

Portions of MI and HI intestine of rainbow trout (*O. mykiss*) (in triplicate from each fish) were processed for histological analyses following Randazzo et al. (2015) and Garcia-Suarez et al. (2018). Sections of 4 μm thickness (cut with a LEICA microtome) were stained respectively with: Mayer's haematoxylin and eosin; Masson's trichrome staining with Aniline Blue solution (Bio Optica), and PAS (Periodic Acid Schiff, Hotchkiss-Mc Manus, Bio Optica). Stained sections were examined under a Zeiss Axio Imager.A2 microscope; images were acquired by mean of a combined color digital camera Axiocam 503 (Zeiss).

2.4. FTIR Imaging measurements and data analysis

Thin sections (~10 μm thickness) of MI and HI intestine of rainbow trout (*O. mykiss*) (in triplicate from each of the five specimens), cut by using a cryotome, were immediately deposited without any fixation process onto CaF_2 optical windows (1 mm thickness, 13 mm diameter), and then air-dried for 30 min (Giorgini et al., 2015a). The infrared analysis of samples was performed within 48 h after cutting at the IR beamline SISSI, ELETTRA – Synchrotron Trieste, using a Bruker VERTEX 70S interferometer coupled with a Hyperion 3000 Vis-IR microscope and equipped with a liquid nitrogen-cooled two-dimensional FPA detector (detector area size 164 × 164 μm, 64 × 64 pixels; Bruker Optics GmbH, Germany). Similar samples prepared by using the same protocol were already tested in our laboratory, evidencing a good stability in time and providing homogeneous and reliable vibrational data sets.

The photomicrograph of each section was collected by using a 15× condenser/objective. This visual inspection let select specific smaller areas representative of whole intestinal mucosa (from the tips of intestinal folds and at least to the underlying submucosa, but excluding the muscular layer). On these areas IR maps were acquired in transmission mode in the MIR range from 4000 cm^{-1} to 800 cm^{-1} (a spectral resolution of 8 cm^{-1} was adopted). IR maps were false color images representing the distribution of the total absorption of the infrared radiation on the mapped area. They had a side of 328 μm and contained 16,384 spectra/pixel with a pixel resolution of ~2.56 μm; each pixel was the result of 256 scans. On each section, a background spectrum was also acquired on a clean portion of the CaF_2 optical window. Raw IR maps were preprocessed with OPUS Atmospheric Compensation routine (to correct the atmospheric contributions of carbon dioxide and water vapor), and then vector normalized on the full frequency range (to avoid artifacts due to local thickness variations) (OPUS 7.1 software package).

To obtain the topographic distribution of lipids, proteins, collagen, mucin and glutamate, preprocessed IR maps were submitted to OPUS 7.1 Integration routine in the following spectral regions: 2995–2816 cm^{-1} (representative of lipids, named LIPIDS); 1726–1481 cm^{-1} (representative of proteins, named PROTEINS);

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