Microorganisms of the intestinal microbiota of *Oncorhynchus mykiss* produce antagonistic substances against bacteria contaminating food and causing disease in humans

Olga Lobos, Andrea Barrera, Carlos Padilla

Department of Microbiology, University of Talca, Talca, Chile

Abstract

The objective was to analyse the antibacterial ability of members of the intestinal microbiota of *Oncorhynchus mykiss* specimens on several bacterial target strains that contaminate food and cause disease in humans. Bacterial colonies from an intestinal portion of the 20 specimens of *O. mykiss* were obtained in different culture media. Several of the colonies showed antibacterial action on different target strains. The bacterial species with the highest antagonistic capacity were *Hafnia alvei* and *Lactococcus lactis* and the more susceptible target strains were *Aeromonas hydrophila*, *Listeria monocytogenes* and *Escherichia coli.* Moreover, it was shown that all the antibacterial substances were susceptible to the action of various proteolytic enzymes. The detection of substances of a proteinaceous nature, possibly bacteriocins produced by bacteria of the intestinal microbiota of *O. mykiss*, allows further study of these products to establish biotechnological developments in the area of health protection and food biopreservation.

Introduction

Currently, foodborne diseases are a growing public health problem worldwide. It is believed that in industrialised countries, over 30% of people suffer from diseases transmitted through food intake (Gálvez *et al*., 2007). At present, there is a tendency for consumers to purchase natural products of high quality that are free of preservatives and chemicals. Incorporating biopreservatives that replace chemical preservatives can be beneficial to human health. For this reason, an intensive search has developed for antibacterial products produced by microorganisms inhabiting different ecological niches (Riley and Wertz, 2002). The result of this research has been the discovery of a large number of proteinaceous antibacterial products called bacteriocins. Bacteriocins have different lethal modes of action on bacteria that degrade food and also on some that are pathogenic to humans (Balciunas *et al*., 2013).

Oncorhynchus mykiss, known as rainbow trout, is a fish of great economic importance worldwide. It is native to the watersheds that drain into the Pacific Ocean in North America, from Alaska to Mexico (FAO, 2005-2012). Major global producers of this fish are in Europe, North America, Chile, Japan and Australia (FAO, 2005-2012). *O. mykiss*, like many other fish species, has a rich intestinal microbiota that, in recent years, has been particularly investigated in relation to its development from youth to adulthood, as has the composition of the microbiota associated with the diet (Ingerslev *et al.,* 2014; Wong *et al.,* 2013; Heikkinen *et al.,* 2006). In general, there are no studies on the antibacterial antagonistic capacity of the intestinal microbiota of *O. mykiss*.

The main objective of this research was to analyse the antibacterial ability of some members of the intestinal microbiota of specimens of *O. mykiss* on various bacterial target strains that can contaminate food and cause disease in humans.

Correspondence: Carlos Padilla, Department of Microbiology, University of Talca, Talca, Chile.
Tel. +56 71 2200451.
E-mail: cpadilla@utalca.cl

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Materials and Methods

Twenty adult males of *O. mykiss,* between 1.3 and 1.6 kg each, were obtained from the fish farming *Panguilemu*, located in the Bío-Bío Region, Chile. The specimens were dissected under sterile conditions to remove the lower digestive tract, and the intestine (5 gr) from each sample was macerated independently until obtaining a homogeneous extract. From each extract, serial dilutions were prepared in sterile distilled water (10^{-1}, 10^{-2}, 10^{-3} and 10^{-5}), and fifty microliters of each dilution were sown onto either MRS agar (Merck, Darmstadt, Germany) Luria-Bertani Agar (Merck) or Tryptic Soy Agar (Merck). The MRS inoculated plates were grown aerobically at 37°C for 18 h to obtain isolated bacterial colonies. To test the antibacterial activity of the selected colonies, three target strains were used: one ATCC and two belonging to the collection of bacterial strains of the Microbiology Department (MD-UTAL) at University of Talca. The ATCC strains used were *Aeromonas hydrophila* ATCC 7966, *Listeria monocytogenes* ATCC 19115, *Salmonella enterica* subsp. Enterica serovar Typhimurium ATCC 14028, *Escherichia coli* ATCC 11229, *Shigella sonnei* ATCC 29930, *Vibrio parahaemolyticus* ATCC 17802, *Staphylococcus aureus* ATCC 29213; while the strains belonging to MD-UTAL were *A. hydrophila*, identified by API Test 20 NE; *S. enterica* subsp. Enterica serovar Typhimurium, *E. coli*, *S. sonnei* and *V. parahaemolyticus* with API 20E; *L. monocytogenes* with Listeria API; and *S. aureus*, with API Staph System.

All target strains were cultured in 2 mL of Soy Broth (Merck) at 37°C for 18 h. Five hundred microliters of each culture were mixed with 7 mL of BHI broth (Merck) plus 0.8% agar-agar, and poured onto the *O. mykiss* isolated intestinal colonies. Plates were incubated at 37°C for 18 h, and colonies producing antagonistic substances detected by the presence of an inhibitory halo around them were isolated and subsequently identified according to the Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2005) and confirmed by various API Tests. After identification of several bacterial species, they were maintained at -85°C in Soy Broth with 15% glycerol. To determine the chemical nature of the antibacterial substances, all antagonistic strains were grown in 10 mL of Tryptic Soy Broth at 37°C for 18 h. After centrifugation at 10,000 g for 20 min, 1 mL of the supernatant from each bacterial strain identified was incubated at 37°C for 2h with 1 mg/mL of the following proteolytic enzymes: papain, pepsin, protease, trypsin and proteinase K (Sigma, St Louis, MO,

USA). On the other hand, to exclude hydrogen peroxide as antibacterial agent, a fraction of the supernatant was treated with catalase (Sigma; 65 IU/mL). In other set of experiments, target strains were cultured in 5 mL of BHI broth (Merck) to achieve a bacterial growth turbidity pattern similar to McFarland No. 0.5 (NCCLS, 2003). Each one of the target strains was then grown in BHI agar plates (Merck). As above, the chemical nature of the antibacterial substance as well as the production of hydrogen peroxide were both studied by adding 20 µL of each supernatant treated with the proteolytic enzymes indicated above or catalase all on the bacterial lawn. To visualise and measure the presence of inhibitory halos, plates were incubated at 37°C for 18 h. In all cases, bacterial cultures without enzymatic treatment were used as controls. To analyse the range of antimicrobial activity, all identified bacterial species with antagonistic capacity were cultured in 5 mL of BHI Broth (Merck) for 18 h at 37°C. Samples were centrifuged at 10,000 *g* for 20 min and as above, target strains were sown, plated with 10 µL of supernatant and incubated at 37°C for 10 h. The diameter of inhibitory halos was evaluated as indicator of antibacterial action.

Results and Discussion

In the intestinal microbiota of 20 *O. mykiss* specimens, the presence of bacterial species with antagonistic capacity against target strains was detected. Seven bacterial species isolated from the intestinal microbiota of fish showed antagonistic activity. In all specimens of *O. mykiss,* unidentified bacterial strains with antagonistic activity were obtained (Table 1). Also, it was observed that the genus *Aeromonas*, represented by *A. veronii* and *A. sobria*, showed the highest percentages of strains with antagonistic capacity (Table 1). All bacterial species detected in the intestinal microbiota of the studied specimens of *O. mykiss* had been described previously (Pond *et al*., 2006; Navarrete *et al*., 2010). It was interesting to note that some genera isolated from the intestinal microbiota presented a broad spectrum of antibacterial action against the target strains studied (Table 2). The obtained bacterial species with a wider spectrum of antibacterial action were *L. lactis* and *H. alvei,* with activity against five target strains, followed by *P. shigelloides,* which was active against four strains. Was observed that *A. hydrophila* presented the greatest antibacterial susceptibility, followed by *L. monocytogenes*. The three target strains of each bacterial species used in this study were susceptible to the antagonistic action of substances produced by bacterial species isolated from the intestine of *O. mykiss.* In addition, it was observed that the same strain with antagonistic capacity may present antibacterial activity on more than one target strain.

Moreover, it was demonstrated that all detected antibacterial substances were susceptible to the action of proteolytic enzymes used in the investigation. It is further noted that the catalase enzyme does not alter the biological activity of the product studied, discarding the presence of hydrogen peroxide as an antibacterial agent.

Considering the peptidic nature of bacteriocins, one can argue that the intestinal microbiota of the studied specimens of *O. mykiss* is composed of bacterial genera with bacteriocinogenic capacity. Similar results have been previously described in which bacterial species isolated from the intestine of *O. mykiss* have lethal action on bacteria such as *Lactococcus garvieae, Aeromonas salmonicida, Streptococcus iniae, Vibrio anguillarum, Vibrio ordalii* and *Yersinia ruckeri* (Brunt *et al*., 2007; Kim and Austin, 2008; Pérez-Sánchez *et al*., 2011), which are pathogenic to salmonids Accordingly, studies on the antagonistic capacity of the intestinal microbiota of *O. mykiss* have been directed towards the control of infectious diseases in salmonids, such as furunculosis and vibriosis (Kayis *et al*., 2009; Beaz-Hidalgo and Figueras, 2012). Usually, these pathologies are treated with antibiotics (Burridge *et al*., 2010).

From the results of this study, it is interesting to note the potential biotechnological use of these antibacterial products, which could be incorporated into human foods

Table 1. Number and percentage of bacterial species obtained from the intestinal microbiota of specimens of *Oncorhynchus mykiss* with antagonistic activity on different target strains.

Bacterial genera	Total (n)	NSA (%)
Aeromonas veronii	12	9 (75)
Aeromonas sobria	8	4 (50)
Plesiomonas shigelloides	14	5 (36)
Shewanella putrefaciens	17	2 (12)
Lactococcus lactis	20	6 (30)
Citrobacter gillenii	12	4 (33)
Hafnia alvei	14	6 (43)
Unidentified	20	9 (45)

NSA, number of strains with antagonistic activity. N=20 specimens of *O. mykiss.*

Table 2. Spectrum of antimicrobial action of the antagonistic substances produced by intestinal microbiota of *Oncorhynchus mykiss.*

Microorganisms with antibacterial activity	N°	Target strains (%)						
		Aeromonas hydrophila	*Listeria monocytogenes*	*Salmonella enterica*	*Escherichia coli*	*Shigella sonnei*	*Vibrio parahaemolyticus*	*Staphylococcus aureus*
Aeromonas veronii	12	8(66)	9(75)	0	0	0	0	7 (58)
Aeromonas sobria	8	4(50)	3(37)	0	0	0	0	3 (57)
Plesiomonas shigelloides	14	0	0	3(21)	5(35)	3(21)	2(14)	0
Shewanella putrefaciens	17	2(11)	0	0	0	0	0	0
Lactococcus lactis	20		5(25)	0	6(30)	3(15)	2(10)	0
Citrobacter gillenii	12	0	0	2(16)	4(33)	4(33)	0	0
Hafnia alvei	14	5(35)	4(28)	2(14)	6(42)	3(21)	0	0
Unidentified	20	7(35)	8(40)	7(35)	9(45)	3(15)	0	0

prone to contamination with microorganisms pathogenic to human beings. In addition, these products could act secondarily as food preservatives.

Considering the chemical nature of antibacterial products elaborated by the intestinal microbiota of *O. mykiss* and according to the results obtained in this study, it is interesting to note that the bacteriocins are closely related to their potential application in the food industry and human health. Bacteriocins possess many positive features, such as a wide range of action, the ability to leave the foods into which they are incorporated unaltered, harmlessness to humans, the capacity to maintain the nutritional value of meals and drinks for a long time and help in the process of digestion of certain foods, among the most important benefits (Cotter *et al*., 2005). The results of this research present the possibility to study new bacteriocins whose application could solve problems of human health and food preservation. Of particular interest are those with lethal capacity against *L. monocytogenes*, *Salmonella* enterica subsp. enterica serovar *Typhimurium* and *S. aureus*, in addition to other susceptible bacterial species. Further studies of these antibacterial products are essential for potential use in the future.

Conclusions

The detection of substances of a proteinaceous nature, possibly bacteriocins produced by bacteria of the intestinal microbiota of *O. mykiss*, offers the possibility to further study these products in order to establish biotechnological developments in the area of health protection and food biopreservation.

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