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Prebiotics and fish immune response: a review of current knowledge and future perspectives

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Prebiotics and fish immune response: a review of current knowledge and future perspectives

Running title: Prebiotics and fish immune response

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Abstract

It is now a well-documented that there is a distinct relation between fish nutrition and health status. Intensification of aquaculture practice to meet market demand causes stress and elevates the risk of the disease outbreak. Therefore, provisions of proper diets as well as appropriate feeding regimens are of high importance in intensive aquaculture. Considering the negative impacts raised by prophylactic and therapeutic use of antibiotic in aquaculture, administration of dietary immunostimulant has been suggested as an alternative to antimicrobial agents. In this sense, functional dietary supplements, including pre-, pro- and synbiotic received increasing attention as an environment-friendly strategy for improving fish health. During the past years, administration of prebiotics in the diet of different fish species revealed promising results in immune response. In the present review article, the topic of the administration of dietary prebiotics in aquaculture is addressed with a special focus on recent findings regarding the effects of prebiotics on fish immune response and possible mode of action. Furthermore, the present study covers the gap in existing knowledge and suggests issues that merit further investigation.

Keywords: Prebiotic, Fish, Nutrition, Immune response

1. Introduction

During the past decade, aquaculture has been considered as one of the fastest-growing industries of food production (Reverter et al. 2014). The elevation of aquatic animal production has been obtained by increasing the production per unit of area which per se results from intensification. However, the elevation of stocking density in aquaculture practices increases the stress level in the cultured organism and the risk of diseases (Pohlenz and Gatlin III 2014). Therefore, disease outbreaks are now considered as one of the most important constraints for development of intensive and super intensive aquaculture systems in many counties (Magnadottir 2010). To resolve the issues raised by intensification, antimicrobial agents have been routinely administered as a preventative means of pathogens control (Cabello 2006). Nevertheless, as a consequence of increasing concerns about negative impacts of chemotherapy on the environment and human health includes the emergence of antibiotic-resistant bacterial strains, accumulation of residual in edible tissues as well as depression of immune system caused the establishment of strict regulation for administration of antimicrobial agents in many countries (Reverter et al. 2014). Therefore, there are increasing interests for using eco-friendly alternatives to the therapeutic use of antimicrobials such as administration of functional dietary supplements (Hoseinifar et al. 2014a). Prebiotics

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"ingredient of the not digestible diet that is beneficial to the host for stimulating selectively the growth and / or the activity of one or more intestinal bacteria" (Gibson 2004) are one of the most studied functional dietary supplements. The studies performed on prebiotics revealed promising results in case of improvement of disease resistance, growth performance, hormonal regulation as well as immune stimulation (Ringø et al. 2014). Considering the importance of nutritional manipulation of fish immune response, in the present review paper, we have covered the effects of prebiotics on fish immune response with a special focus on recent advances, mode of action as well as research gaps and future perspectives. The results cited and discussed in the present review include studies published in peer-reviewed scientific journals, open access peer-reviewed scientific journals, scientific books and abstracts presented in proceeding books from international conferences.

2. Crosstalk between immune system and diet

The term immunity is used to designate immune reaction against foreign agents, including microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and macromolecules (proteins and polysaccharides) without pathological consequences. The fish immune response (see Manning 1998) starts with the humoral (complement system, lysozyme, acute phase proteins, antimicrobial peptides, interferon (IFN), lectins, proteases, protease inhibitors or eicosanoids) and cellular (monocyte-macrophages, granulocytes, natural killer (NK) and nonspecific cytotoxic (NCC) cells) components of the innate immune system after coming into contact with structures of the pathogen known as pathogen-associated molecular patterns (PAMPs), which are common molecules not typically found in eukaryotic cells, such as viral double-stranded RNA, bacterial lipopolysaccharide (LPS) and certain sugars. This response usually starts immediately and lasts several hours. The antigen is then processed and presented to the adaptive immune system components (B and T lymphocytes), which elaborates the adaptive or specific response (immunoglobulins (Ig) and cytotoxic T lymphocytes (CTLs). This takes several days but, due to the lack of thermoregulation, the response achieved is never comparable in terms of effectiveness with the mammalian response. The control and integration of this immune response is carried out by cytokines, which are mainly produced by lymphocytes and monocyte/macrophages after stimulation.

The fish immune response is modulated by many intrinsic and extrinsic factors, including environmental factors (temperature, salinity, photoperiod, etc.) and physiological status (nutrition, age, reproductive cycle, hormonal balance, stress, etc.). Among them, the use of immunostimulants, mainly from natural sources, is a very practical

approach to improve the success of the aquaculture because they increase the fish health reducing the impact of diseases and stress (Sakai 1999; Bricknell and Dalmo 2005; Kiron et al. 2012). Nowadays, an immunostimulant is defined as a **naturally-occurring** compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens. **The administration of immunostimulants** depends **upon** the fish size but bathing for larvae and oral routes for juveniles and adults **is** the preferred **route** by farmers since avoid fish handling and stress. **Therefore**, most of the available information has confirmed the beneficial effects of immunostimulants on humoral and cellular immunity in blood, head-kidney or spleen tissues, gene expression or disease resistance after oral administration as dietary supplements (Sakai et al. 1999; Bricknell and Dalmo 2005; Kiron et al. 2012). These immunostimulants include nucleotides, vitamins, fatty acids, yeast and bacteria products (beta-glucans, chitin, RNA, etc.), plant extracts, probiotics and prebiotics. Strikingly, the physiological responses, including immunity, after oral administration of immunostimulants in the target tissue, the gastrointestinal tract, have been not widely analyzed until the last decade. Thus, in this section, we will focus on the changes in the gut after dietary administration of immunostimulants, with special emphasis to the immunity after prebiotic administration.

Apart from the typical fish immune organs (head-kidney, spleen and thymus), which have received most of the attention during several decades, the fish immune system also consists on physical barriers, which have attracted our interest more lately. These barriers are composed of the epithelia, and their mucus secretions, forming the mucosa-associated lymphoid tissue (MALT) (Rombout et al. 2011). In fish, the MALT is composed by populations of dispersed **cells**, including T and B lymphocytes, macrophages, plasma cells, granulocytes and mast cells. According to anatomical location, the MALT in teleost fish is subdivided into gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT) and skin-associated lymphoid tissue (SALT) (Salinas et al. 2011). In general, the fish MALT constitutes a very large area for the possible microbial invasion (Wilson and Laurent 2002) and contains defence mechanisms (both innate and adaptive) that constitute the first line of defence against a broad spectrum of pathogens present in the aquatic environment (Magnadottir 2010; Rombout et al. 2011). Functions of this system appear to be related with to the ability to trap antigens and release IgT and IgM involved in responses against several pathogens (Zhang et al. 2010; Salinas et al. 2011; Xu et al. 2013). Thus, further investigations in the immunological characterization of MALT and its role in disease resistance are in progress and attracting great interest.

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Contrary to mammals, fish lacks lymph nodes, M cells or IgA secretion in the gut. However, intraepithelial (IELs) and lamina propria (LPLs) leucocytes are diffusely found in fish, including B and T lymphocytes, macrophages and eosinophilic and neutrophilic granulocytes (Rombout et al. 2011) forming the GALT. In the gut mucus layer, immune molecules such as complement, lysozyme, proteases, antiproteases, antimicrobial peptides and immunoglobulins have been also identified. In fact, IgT was discovered in 2005 and seems to be the mucosa-specialized Ig in fish (Zhang et al. 2010; Salinas et al. 2011; Xu et al. 2013) playing a similar role than the mammalian IgA. Unfortunately, there is not much information about the GALT immunological functions in fish after dietary administration of immunostimulants. This field almost emerged at the same time than the interest for probiotics, but the cellular and molecular mechanisms remained still unknown (Nayak 2010; Lazado and Caipang 2014). Afterwards, the use of prebiotics as a way to improve the fish health has also attracted the researchers and farmers interests. In this aspect, the use of prebiotics, alone or in combination with probiotics, as immunostimulants has demonstrated to increase the fish immune response and disease resistance (Ringø et al. 2010; Song et al. 2014; Torrecillas et al. 2014). However, at this respect, most of the studies include the characterization of the gut microbiota and morphology after prebiotic administration, but the characterization of the GALT presence and functioning is scarcely described. Therefore, comprehensive description of the mechanisms used by prebiotics to stimulate the immune response, at the local and systemic level, are still waiting.

Most of the information regarding the role of prebiotics in the gut physiology includes the study of the microbiota. These microbial communities in the gut varied with the prebiotic used (nature, concentration, duration, etc.) and the fish species. In general, the dietary intake of prebiotics provokes in the microbiota a reduction in variability with a higher number of “good” bacteria (namely *Lactobacillus* and *Bifidobacterium* species) and lower of “bad” bacteria (potential pathogenic bacteria such as *Aeromonas* spp. or *Vibrio* spp.) (Ringø et al. 2010; Song et al. 2014; Torrecillas et al. 2014). In this case, most of the effects of prebiotics on immunity are indirect and attributed to the change in the gut microbiota which are the direct players in the improvement of the immunity. Thus, these beneficial bacteria help in feed conversion efficiency, increased fish growth and confer protection against pathogens by competitive exclusion for adhesion sites, production of organic acids (formic acid, acetic acid, lactic acid), hydrogen peroxide and several other compounds such as antibiotics, bacteriocins, siderophores, lysozyme and also modulate physiological and immunological responses in fish (Nayak 2010). These prebiotics may also interact with fish pattern recognition receptors (PRRs) in the form of microbe-associated molecular patterns (MAMPs) such as

135 teichoic acid, peptidoglycan, glycosylated protein, or the capsular polysaccharide of bacteria (Bron et al. 2012).
136 PRRs have been identified in teleost fish, including toll-like receptors (TLRs), NOD-like receptors (NLR), C-type
137 lectin receptors (CLRs) and peptidoglycan recognition proteins (PGRPs) (Boltaña et al. 2011). In this sense,
138 probiotics have shown to recruit lymphocytes and granulocytes to the gut as well as regulate the gene expression to
139 favor the immune response by attracting leucocytes and decreasing inflammation (Picchietti et al. 2007, 2009; He et
140 al. 2013; Cerezuela et al. 2013b). In addition, administration of prebiotics may result in a higher production by the
141 microbiota of metabolites such as propionate, butyrate or short-chain fatty acids (SCFA), which can be used by the
142 immune cells of the GALT, as reported in mammals (Bach Knudsen et al. 2003), and be activated by specific
143 SCFA-receptors. Though these compounds have been demonstrated to be produced in the fish gut after prebiotic
144 administration (Mahious et al. 2006b; Rurangwa et al. 2008; Geraylou et al. 2012) the presence of such a SCFA-
145 receptor has not been confirmed in fish.
146 Another aspect that is seriously affected by prebiotics is the gut histomorphology. In this sense, most of the studies
147 have focused and documented changes in the villi and microvilli (number, length, width, etc.), number of mucous-
148 producing cells, abundance of the mucus layer, leucocyte infiltration, etc. after prebiotic administration to fish
149 (Ringø et al. 2010; Torrecillas et al. 2014). Apart from the nutritional considerations of such changes, they affect to
150 the ability of pathogens to adhere and translocate the intestinal barrier and colonize internal tissues. In this sense, the
151 mucus production is increased in inulin-fed gilthead seabream specimens, even when the number of goblet cells was
152 decreased (Cerezuela et al. 2013a), and in MOS-fed rainbow trout (Rodrigues-Estrada et al. 2008). Moreover, gut
153 enterocytes appear more tightly adhered. European sea bass fed with MOS resulted in lesser disruption of tight
154 junctions (TJs) and a better-preserved cytoarchitecture of the intestinal barrier as evidenced by the electron
155 microscopy observations (Torrecillas et al. 2013). This was also confirmed at gene level in seabream fed inulin,
156 which showed up-regulation of the gut transcripts of occludin, a protein involved in the tight junctions (Cerezuela et
157 al. 2013b) in a similar way as it occurs in mammals (Cani et al. 2009). These two factors greatly contribute to
158 difficult the bacterial translocation and have an indirect effect on immunity and disease resistance by hampering the
159 pathogen entry, colonization and dissemination.
160 There is limited information about the proper immunological role of prebiotics at the administration site, the gut and
161 GALT. In the table 1, we have summarized the scarce findings regarding the GALT presence and function in fish
162 fed prebiotics, among which only inulin and MOS have attracted some interest. Overall, together to elevation of

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3 163 mucus production, prebiotics increase the bactericidal and lysozyme activities (Torrecillas et al. 2011, 2013) making
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5 164 the first line of defence more robust. Afterwards, bacteria that can still pass it find a more well-structured and
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7 165 resistant line formed by the epithelial cells (Torrecillas et al. 2013; Cerezuela et al. 2013b). Just in case of bacterial
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9 166 translocation, they will encounter a higher number of intraepithelial and lamina propria leucocytes that could
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11 167 recognize, bind and eliminate them. The mechanisms used by prebiotics to enhance the gut and systemic immunity
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13 168 remained unknown but several hypotheses may exist: i) GALT leucocytes can directly contact with the luminal
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15 169 prebiotics and be activated, ii) enterocytes metabolism may mediate immune activation, iii) changed microbiota is
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17 170 responsible for the immune activation, and iv) prebiotics may cross the epithelia and encounter to leucocytes.
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19 171 Firstly, intraepithelial leucocytes might cross the epithelial border and be in direct contact of the gut luminal
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21 172 substances, including prebiotics, working as substitutes of the inexistent fish M cells. If this happens, which has not
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23 173 been confirmed by electron microscopy, leucocytes could recognize and directly bind to the prebiotic saccharides by
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25 174 membrane PRRs and become activated. In the case of inulin, it is known that long-chained inulin stimulates the
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27 175 human immune system by binding to specific lectin-like receptors on leucocytes and inducing macrophage
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29 176 proliferation (Seifert & Watzl 2007) though this has not been confirmed in fish. Thus, seabream leucocytes directly
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31 177 exposed to inulin failed to change phagocyte-related immune functions suggesting the absence of such receptors
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33 178 (Cerezuela et al. 2008). For MOS, mannose mainly binds to the mannose-receptor (MR) present in macrophages,
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35 179 endothelial cells and immature dendritic cells but can also be recognized by CD209 (DC-SIGN) and dectin-2
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37 180 receptors. The existence of mannose receptors and CD209 has been confirmed in teleost fish (Rodríguez et al. 2003;
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39 181 Ordás et al. 2011) but its binding capacity to prebiotics has not evaluated. No receptors for FOS have been reported
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41 182 in any animals studied, including fish. Secondly, as evidenced by morphological and functional studies, prebiotics
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43 183 alter the enterocyte morphology and physiology, and they could then produce factors that increase the leucocyte
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45 184 recruitment to the intestine mucosa and their immune functions. For example, enterocytes are able to translocate
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47 185 particles and bacteria from the mammalian gut lumen by different mechanisms as well as express different PRRs
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49 186 including TLRs and act as antigen-presenting cells (Miron and Cristea 2012). In fish, enterocytes are able to
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51 187 translocate several substances from the luminal part to inner tissues and blood but the ability to translocate bacteria
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53 188 to the phagocytic cells remained unclear (Rombout et al. 2011). No study has evaluated the enterocyte expression of
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55 189 immune-related genes or proteins. Thirdly, prebiotics affect the microbiota which per se, or their products, increases
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57 190 the local and systemic immunity. This is so far the most explored hypothesis, but it is not well known if GALT
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leucocytes can cross the epithelial border and directly recognize the MAMPs or they are activated by microbiota products. **Fourth**, and lastly, it is not known if prebiotics could cross the gut epithelia and encounter the GALT leucocytes. For instance, complex structures such as poly D, L-lactide-co-glycolic acid (PLGA) microparticles are able to cross the intestinal barrier (Ellis 1998; Tian et al. 2008) but this has not been confirmed for prebiotics. Further studies are still needed to understand and ascertain the role played by prebiotics in GALT **immunity**, and the mechanisms involved.

Finally, the difficulty to isolate GALT leucocytes with intact functions and the lack of leucocyte markers (antibodies or gene markers) makes this progress more difficult. **Hence**, the available approach is the use of gene expression. **There are some published studies which** documented the gut expression of immune-related genes after prebiotic feeding (Cerezuela et al. 2013a, b; Lokesh et al. 2012). They seem to indicate that the immune **stimulation** and the leucocyte recruitment into the gut mucosa **are** not due to an inflammation process. Massive sequencing projects in fish have been undertaken in the past years. Unfortunately, very few have looked at the gut (Doñate et al. 2010; Skugor et al. 2011; Calduch-Giner et al. 2012). These studies have revealed some immune-related **genes**, but deeper characterization about the precise expression and functions would help to understand the gut physiology, and of GALT in particular. For example, the precise repertoire and functions of fish enterocytes in immunity as well as the presence, distribution and functions of the scattered leucocytes should be investigated in the future. This information would help in the design of diets with balanced and better immunological properties. Moreover, this will be also important **for** the design and generation of oral vaccines, a field under necessary development in fish and with great interest in aquaculture.

3. Prebiotics and humoral immune response

3.1 Mannan oligosaccharides (MOS)

Mannan oligosaccharides (MOS), glucomannoprotein-complexes derived from the cell wall of yeast (*Saccharomyces cerevisiae*), are one of the most common prebiotic evaluated in fish (Merrifield et al. 2010) (Table 2). Staykov et al. (2007) observed that diet containing 2 g kg⁻¹ MOS improved the lysozyme activity, the alternative complement pathway activity (ACPA) and the classical complement pathway activity (CCPA) in rainbow trout (*Oncorhynchus mykiss*). Talpur et al. (2014) reported that dietary supplemented with 2 g kg⁻¹ MOS for 12 weeks improved the lysozyme activity of snakehead (*Channa striata*). In line with the previous studies, improved lysozyme activity was

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observed in red drum (*Sciaenops ocellatus*) fed the diet containing a relatively high level of MOS (10 g kg⁻¹) for 8 weeks (Zhou et al. 2010) or **six** weeks (Buentello et al. 2010). However, diet containing 2-6 g kg⁻¹ MOS had no significant impact on the lysozyme activity of European sea bass (*Dicentrarchus labrax*) (Torrecillas et al. 2007, 2011). Dietary supplementation of 2 g kg⁻¹ MOS was evaluated in channel catfish (*Ictalurus punctatus*) (initial weight, 10.6 g) (Welker et al. 2007), and the results showed that inclusion of MOS did not affect the immune function. Interestingly, the same research group reported that the same dose of MOS exerted no significant effect on the lysozyme activity and SH50, while significantly increased the antibody titre of channel catfish (initial weight, 39.1 g) (Welker et al. 2012). Interestingly, decreased lysozyme activity was observed in Atlantic salmon (*Salmo salar*) fed the diet containing 10 g kg⁻¹ MOS for **four** months (Grisdale-Helland et al. 2008). In fact, this **long-term** administration of MOS in fish has been questioned previously (Yoshida et al. 1995). Therefore, further studies are needed to determine the appropriate inclusion level of MOS and duration of administration in fish.

3.2 Fructo-oligosaccharides (FOS)

Fructo-oligosaccharides (FOS) refers to short and medium chains of β-D-fructans in which fructosyl units are bound by β-(2-1) glycosidic linkages and attached to a terminal glucose unit. It is assumed that there are no specific cellular FOS receptors in vertebrates. As such it is rather speculative to assert any immunological effects by direct action of FOS on host cells (Ringø et al. 2010). Alternatively, dietary inclusion of FOS could positively shape the gut microbial community and then modulate the immune function of fish (Mahious et al. 2006a; Ringø et al. 2010). Although the mechanisms are not clear, several studies have shown that FOS could improve the humoral immune response of fish (Table 3). For example, improved lysozyme activity was observed in red drum fed the diet containing 10 g kg⁻¹ FOS for six weeks (Buentello et al. 2010) or eight weeks (Zhou et al. 2010). Soleimani et al. (2012) investigated the effect of FOS on the innate immune response of Caspian roach (*Rutilus rutilus*) fry. At the end of the study (7 weeks) the innate immune responses (Ig level, lysozyme activity and ACH50) were significantly higher in 2 g kg⁻¹ and 3 g kg⁻¹ FOS fed fish, whereas, 1 g kg⁻¹ dietary FOS only elevated serum lysozyme activity. Akrami et al. (2013) reported that dietary supplementation of high level of FOS (10 and 20 g kg⁻¹) for 11 weeks improved the lysozyme activity of stellate sturgeon (*Acipenser stellatus*). However, Ai et al. (2011) observed that dietary supplementation of 2 and 4 g kg⁻¹ FOS for 10 weeks had no significant **remarkable** on the lysozyme activity and ACH50 of large yellow croaker, *Larimichthys crocea*. **Likewise**, Ye et al. (2011) reported that diet containing 5 g kg⁻¹ FOS had no significant impact on the lysozyme activity in Japanese flounder (*Paralichthys olivaceus*).

Furthermore, administration of 10, 20 or 30 g kg⁻¹ FOS had no remarkable effects on growth performance and haematological parameters of common carp (*Cyprinus carpio*). However, immunological parameters (WBC and respiratory burst activity) as well as intestinal microbiota (LAB levels) were affected by dietary FOS (Hoseinifar et al. 2014c). This contradictory result may be attributable to different dosage, different duration of FOS administration, different fish species or life stage (Hoseinifar et al. 2010).

3.3 Inulin

Inulin is a term applied to a heterogeneous blend of fructose polymers differing from FOS (or oligofructose), a subgroup of inulin, by the degree of polymerisation (DP): inulin DP > 10 and FOS DP ≤ 10 (Niness 1999). Several studies have evaluated the effect of inulin supplemented diet on the humoral immune response of fish (Table 4). It has been reported that 10 g kg⁻¹ of inulin showed no significant impact on the complement level and peroxidase activity of gilthead seabream (*Sparus aurata* L.) with initial body weight 150 g (Cerezuela et al. 2008, 2012a) or with initial body weight 100 g (Cerezuela et al. 2012a). However, the same research group observed that dietary supplemented with 10 g kg⁻¹ of inulin could significantly improve the complement activity and IgM level of gilthead seabream (initial body weight 50 g) (Cerezuela et al. 2012b). Diet containing 5 g kg⁻¹ inulin significantly improved the lysozyme activity of Nile tilapia (*Oreochromis niloticus*) (Ibrahim et al. 2010) and the total immunoglobulin of hybrid surubim (*Pseudoplatystoma* sp.) (Mouriño et al. 2012). In line with previous studies, Reyes-Becerril et al. (2014) reported that the lysozyme activity, IgM level and myeloperoxidase activity elevated significantly in leopard grouper (*Mycteroperca rosacea*) fed with diet supplemented with 5 g kg⁻¹ inulin for 8 weeks.

4.4 Other prebiotic

Galactooligosaccharides (GOS) consisting of 2-20 molecules of galactose and glucose and can be produced through enzymatic treatments of lactose (Ringø et al. 2010; Kiron 2012). The prebiotic has been widely been used in endothermic animals, however, few studies have been carried out using GOS in fish (Zhou et al. 2010; Grisdale-Helland et al. 2008; Hoseinifar et al. 2013) (Table 5). According to Zhou et al. (2010), diet administration of 10 g kg⁻¹ GOS for eight weeks significantly improved the lysozyme activity of red drum. On the contrary, decreased lysozyme activity was observed in Atlantic salmon fed diet containing 10 g kg⁻¹ GOS for four months (Grisdale-Helland et al. 2008).

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3 274 Arabinoxylan-oligosaccharides (AXOS), the hydrolysis products of arabinoxylans (AX), are newly discovered class
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5 275 of candidate prebiotics that exerted different properties depending upon their structure (Ringø et al. 2010). Recently,
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7 276 Geraylou et al. (2012) evaluated the effects of two different structures of AXOS, namely AXOS-32-0.30 (average
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9 277 degree of polymerization: 32, average degree of substitution: 0.30) and AXOS-3-0.25, on the growth performance,
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11 278 immune responses, gut microbial fermentation and gut bacterial composition of juvenile Siberian sturgeon
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13 279 (*Acipenser baerii*). The ACH50 and total peroxidase enzyme concentration (PO) in serum of fish fed a diet
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15 280 containing AXOS-32-0.30 were substantially higher than of fish fed the control diet or AXOS-3-0.25, while the
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17 281 serum lysozyme activity was not affected by AXOS. Subsequently, the same research group reported that diet
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19 282 containing 10 g kg⁻¹ AXOS-32-0.30 remarkably improved the ACH50, but had no significant impact on the serum
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21 283 peroxidase and total immunoglobulin of Siberian sturgeon (Geraylou et al. 2013).
22
23 284 Levan, another polymer of fructose, is being considered as an ideal prebiotic and immunonutrient in aquaculture
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25 285 (Gupta et al. 2014). Levan when incorporated at 5 and 10 g kg⁻¹ in the feeds of juvenile common carp *Cyprinus*
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27 286 *carpio*, improved the serum lysozyme activity (Rairakhwada et al. 2007). The juveniles of another carp variety, *L.*
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29 287 *rohita* were found to have high serum lysozyme activity and highest relative survival percentage against *Aeromonas*
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31 288 *hydrophila* upon levan inclusion in the feed at 12.5 g kg⁻¹ (Gupta et al. 2008). In a recent investigation, serum
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33 289 lysozyme activity and survival rate of orange-spotted grouper (*Epinephelus coioides* H.) after challenge with *Vibrio*
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35 290 *harveyi* were significantly higher in the 25 g kg⁻¹ levan supplemented group compared to the control group (Huang
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37 291 et al. 2014).
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39 292 Chitooligosaccharide (COS) is a type of oligosaccharides, which is obtained by chemical and enzymatic hydrolysis
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41 293 of chitosan. The COS has a higher activity and more physiological functions than chitosan due to its lower
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43 294 molecular weight or its ready solubility in water. COS when incorporated at 2 g kg⁻¹ in the feeds of koi (*Cyprinus*
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45 295 *carpio koi*), improved the serum SOD and lysozyme activity (Lin et al. 2012).
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47 296 3.5 Commercial prebiotic
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49 297 Yeast by-products from the brewing industry are natural prebiotic additives that have been shown to positively
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51 298 influence the growth performance and immune responses of some fish species (Ringø et al. 2010) (Table 6). Li and
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53 299 Gatlin have conducted a series of studies to evaluate the application of a commercial prebiotic (Brewtech®), partially
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55 300 autolyzed brewer's yeast, in hybrid striped bass (*Morone chrysops* × *M. saxatilis*) with different initial weight. The
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57 301 results showed that the inclusion of 10, 20 g kg⁻¹ of Brewtech® did not affect the serum lysozyme activity of hybrid
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striped bass with initial weight of 9.7 g (Li and Gatlin 2003) and 19.7 g (Li and Gatlin 2004). However, the same dose of Brewtech[®] improved the serum peroxidase activity of hybrid striped bass with initial weight of 64.5 g and 118 g (Li and Gatlin 2003). Another common used commercial prebiotic, derived from the cell wall of brewer's yeast and contained MOS and β -glucans, has been reported to elevate the serum lysozyme and complement activities of rainbow trout (Yar Ahmadi et al. 2014a). **In contrast**, yeast cell wall originated commercial prebiotic had no significant impact on the serum lysozyme activity and IgM level of great Sturgeon (*Huso huso*) (Ta'ati et al. 2011), and on the IgM level and complement component **three levels** of Japanese seabass (*Lateolabrax japonicus*) (Yu et al. 2014). Grobiotic[®]-A, a mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products has been extensively studied (Ringø et al. 2010). To our knowledge, however, **all the studies have shown** that Grobiotic[®]-A has no significant impact on the humoral immune response in different fish species, such as hybrid striped bass (Li and Gatlin 2004, 2005), Golden shiners (*Notemigonus crysoleucas*) (Lochmann et al. 2009) and Nile tilapia (Zheng et al. 2011). Furthermore, in a study with rainbow trout, the effect of Vitacel[®] (a commercial prebiotic mainly consists of cellulose and hemicellulose) was studied on humoral immune parameters as well as immune gene expression (Yarahmadi et al. 2014b). The results revealed that administration of 10 g kg⁻¹ Vitacel[®] remarkably elevated serum innate immune parameters include serum lysozyme, ACH50, bactericidal activity and agglutination antibody titer. Also, lysozyme and TNF α genes expression were significantly upregulated in the head kidney of Vitacel[®] fed rainbow trout.

Though some of the prebiotic products may have the capacity to positively influence the immunity of fish, the information available at this point is relatively limited. Therefore, the reviews mentioned earlier suggested that further studies are needed to fill the knowledge gap (Kiron 2012; Song et al. 2014).

4. Prebiotics and cellular immune response

There are few previous reviews regarding the effects of prebiotics in **aquaculture**, and most of them focus on their effects on the gastrointestinal tract (see Ringø et al. 2010; Song et al. 2014). Prebiotics, such as fructooligosaccharide, mannanoligosaccharide, inulin, or β -glucan, sometimes are called immunosaccharides because they can directly enhance different innate immune responses. Nevertheless, other results seem to indicate that not all the prebiotics has immunostimulant properties. For example, there is only one available paper focus on

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the effects of IMO (a mixture of isomaltose, isomaltotriose, panose, isomaltotetraose, etc.) on aquatic animals and no clear effect was noted on the immune response (Li et al. 2009).

Most available studies focus on the effects of different prebiotics on humoral immune response while there are only few papers focus on their effects on cellular immune response, mainly phagocytosis and respiratory burst. Regarding the effects of prebiotics on phagocytosis contradictory results have been obtained although they seem to suggest that numerous factors (both intraspecific and interspecific ones) are involved. For example, while the phagocytic activity increase after a 12-week feeding test of rainbow trout (*Salmo gairdneri*) fed diets with two inclusion levels (0.25% and 0.5%) of MOS, the observed increments were only significant in the group fed the highest MOS concentration, respect to the values for control group (fed non-supplemented diet) (Rodriguez-Estrada et al. 2013). However, in another study carried out with Japanese flounder (*Paralichthys olivaceus*) fed different experimental diets (control or non-supplemented, 2.5 and 5 g kg⁻¹ FOS and + 2.5 and 5 g kg⁻¹ MOS) for eight weeks, the results pointed that none of the diets altered the phagocytic activity of blood leucocytes (Ye et al. 2011). Perhaps, the reason of observing an immunostimulant effect on phagocytosis is linked with the origin of those leucocytes. No effect was observed when blood leucocytes were used while a positive effect was obtained when the phagocytic cells were obtained from the head-kidney (the haemopoietic organ). Further studies are needed to demonstrate or not this hypothesis.

Inulin (a water-soluble carbohydrate, a fructooligosaccharide, FOS) is a prebiotic that plays an important role in the immune function in mammals, although its effects on fish are not similar, regarding the available results. Even though inulin is not a natural fiber in fish diets, inulin may have interesting applications in aquaculture to stimulate the good gut bacteria, suppress pathogens and enhance immune response (Ringø et al. 2010). Furthermore, it is known that inulin, particularly long chain molecules, stimulates the human immune system by binding to specific lectin-like receptors on leucocytes and increasing macrophage proliferation (Causey et al. 1998). Moreover, mice fed inulin show an increased percentage of NK cells and/or macrophage response kinetic (Kelly-Quagiliana et al. 1998). The effect of this prebiotic on gilthead seabream (*Sparus aurata* L.) has been studied both *in vitro* and *in vivo* and results support that inulin is not an immunostimulant for this fish species. Seabream head-kidney leucocytes were incubated with inulin (ranging from 0 to 1000 mg ml⁻¹) for different times (ranging from 30, 90, 180 and 300 min to 24 h) and any effect was observed on the main innate cellular immune responses (phagocytic, respiratory burst and natural cytotoxic activities and leucocyte peroxidase content). These results seem to indicate that inulin has no direct effect on seabream leucocytes, perhaps because they do not have a receptor for this carbohydrate

(Cerezuela et al. 2008). For the in vivo study, seabream specimens were fed for **one or two** weeks with a commercial diet supplemented with inulin [0 (control), 0.5 and 1% of inulin]. In this case, dietary administration of inulin to seabream specimens produced a significant inhibition in phagocytosis and respiratory burst of head kidney leucocytes from specimens fed inulin diets for **one** week (Cerezuela et al. 2008). These results give rise to another work in order to evaluate the effects of inulin on the same tested cellular immune parameters of gilthead seabream, but using higher concentrations of inulin (10, 15 and 30 g kg⁻¹) in order to determine the optimal concentration for stimulating the seabream's immune system. The optimum concentration of inulin was 10 g kg⁻¹ because it produces (after **two** weeks of administration) stimulation of the serum complement activity and the IgM level, as well as leucocyte phagocytic and respiratory burst activities (Cerezuela et al. 2012). It is important to know that, although it has been demonstrated that inulin **suppresses** pathogens and enhance **immunes** response in fish, high inulin dosages might have a **negative** impact in gut morphology (Ringø et al. 2010), for this reason it seems to be imperative to adjust the optimum dosage without damage the fish gut.

Dietary supplementation of 2 g kg⁻¹ of MOS (glucomannoprotein complexes derived from the cell wall of the yeast *Saccharomyces cerevisiae*) was evaluated in channel catfish (*Ictalurus punctatus*) and did not affect growth immune function (Welker et al. 2007). Furthermore, serum bactericidal activity was not affected by the 2 g kg⁻¹ MOS treatment (MOS derived from the outer cell wall of *S. cerevisiae* strain 1026) (Staykov et al. 2007). The originality of these experimental trials was to demonstrate the ability of MOS to improve the growth performance, survival, and immune status of rainbow trout produced in net cages or raceways although any cellular immune response was tested.

However, when AXOS preparations [AXOS-32-0.30 (average degree of polymerization: 32, average degree of substitution: 0.30) and AXOS-3-0.25] were administered at 2% of diet to juvenile Siberian sturgeon (*Acipenser baerii*) significantly enhanced the phagocytic activity of fish macrophages **compared with** the control group (Geraylou et al. 2012). Synergistic effects of AXOS and probiotic (*L. lactis* ST G45, a putative endogenous probiotic) diets were only observed for phagocytic and alternative complement activity (Geraylou et al. 2013). The respiratory burst activity of leucocytes from stellate sturgeon (*Acipenser stellatus*) juvenile was not significantly affected by dietary 1% FOS ($P < 0.05$) (Akrami et al. 2013). **In contrast**, Hoseinifar et al. (2014c) reported significant elevation of respiratory burst activity of leucocytes in common carp fry fed dietary FOS.

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3. Mode of action of prebiotic on fish immune system

Regarding the course of action, several and multiple effects have been demonstrated. Prebiotics can be considered as a beneficial dietary supplement for improving growth performance, boosted immune responses, increased stress resistance, improving digestive enzyme activities. To our days, most of the studies carried out on the use of dietary prebiotics on fish have been focused upon the determination of parameters related to digestive or immune functions. Several studies analysed the survival of the specimens after fed the prebiotic. The results demonstrated that usually prebiotic improve the growth factors (daily weight gain, final weight, weight gain, specific growth rate, condition factor, food conversion ratio, feed efficiency ratio and protein efficiency ratio) (Xu et al. 2008; Gültepe et al. 2012; Hoseinifar et al. 2013) or not produce any effect on them (Burr et al. 2010; Razeghi Mansour et al. 2012; Ebrahimi et al. 2012; Hoseinifar et al. 2014c). This could be explained taken into account that the effects of prebiotics may vary depending upon the fish species analysed. A study carried out by Razeghi Mansour and coworkers (2012) suggested that the prebiotic mannan oligosaccharide did not influence the growth performance in giant sturgeon juvenile, and authors pointed out that it is not appropriate for supplementation its diet.

Regarding the hematological parameters, perhaps the most studied parameters after the administration of prebiotics in fish, many different ones have been analysed, including blood cell counts (red blood cells, white blood cells, thrombocyte counts or lymphocyte levels), erythrocyte-related parameters (e.g., mean corpuscle volume, hematocrit, hemoglobin) and serum activities [e.g. enzyme activities (lactate dehydrogenase alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, phenoloxidase activities), metabolic products (cholesterol, glucose and total proteins)] (Hoseinifar et al. 2011; Ebrahimi et al. 2012; Zhang et al. 2013). However, not always increments in such haematological parameters have been recorded (Gültepe et al. 2012).

Among the immune parameters, some of them have been studied although usually focus on humoral immune activities [such as serum immunoglobulin levels, lysozyme activity and alternative complement activity (ACH50)] (Geraylou et al. 2012; Akrami et al. 2013; Soleimani et al. 2012).

Other important effects of prebiotics are changes or alteration in the fish gut, either on gut morphology or in the gut microbial community (both diversity and count). Furthermore, sometimes, beneficial effects on gut have been due to the prebiotic consumption (Anguiano et al. 2013). Similarly, increments in the digestive enzyme activities (amylase, lipase and protease) (Xu et al. 2008; Soleimani et al., 2012; Eshaghzadeh et al. 2014) or in liver enzyme activities [superoxide dismutase (SOD) activity] (Zhang et al. 2013) have also been demonstrated as a consequence of the

administration of diet enriched with prebiotics. Perhaps such increments of enzymes are correlated with the observed weight gain and feed utilization and efficiency in those fish fed prebiotics. However, not always the increments of digestive enzymes were associated to the prebiotics administration (Anguiano et al. 2013). On the other hand, inulin altered the gut mucosa of gilthead seabream. **In fact**, a structural and ultrastructural study revealed that, although the intestinal absorptive area was not altered, different signs of gut oedema and inflammation that could compromise the body homeostasis (Cerezuela et al. 2013). Furthermore, inulin causes important alterations in the intestinal microbiota by significantly decreasing bacterial diversity, as demonstrated by the specific richness, Shannon, and range-weighted richness indices (Cerezuela et al. 2013). **Also**, administration of XOS in fry diet significantly elevated the autochthonous lactic acid bacteria (Hoseinifar et al. 2014b). Especially increase in Bifidobacteria, which is produced by certain prebiotics (Roberfroid et al. 2010) can be regarded as a marker of intestinal health.

Till present, only few studies focus on the effect of prebiotic on the stress tolerance; for instance, resistance to salinity stress (150 g l⁻¹) (Soleimani et al. 2012; Hoseinifar et al. 2014c). Similarly, some works have demonstrated an increase in the disease resistance in certain fish species. For example, *C. carpio* fingerlings (Ebrahimi et al. 2012) and triangular bream (*Megalobrama terminalis*) specimens (Zhan et al. 2013) to *A. hydrophila* infection after feeding prebiotics. **More recently, the effects of prebiotics on mucosal immunity are been considered. The results revealed that XOS significantly increased skin mucus antibacterial activity and protein levels (Hoseinifar et al. 2014b).**

Regarding **the course of action** of prebiotics, it has been proposed that immunosaccharides directly activate the innate immune system by interacting with pattern recognition receptors (PRR) expressed on innate immune cells (such as beta-glucan receptors or dentin-1 receptors that are expressed on macrophages) (Brown et al. 2002) and this ligand-receptor interaction activates signal transduction molecules such as NF-kB, that stimulate immune cells (Yadav and Schorey 2002). Furthermore, they can also be recognized by **a microbe associated** molecular patterns (MAMPs) (such as teichoic acid, peptidoglycan, glycosylated protein, or the capsular polysaccharide of bacteria, triggering an immune response) (Bron et al. 2012; Song et al. 2014). Thus, it appears that prebiotics activate the innate immune system in two ways: (1) by directly stimulating the innate immune system, or (2) by enhancing the growth of commensal microbiota (Song et al. 2014). New studies will indicate even some other way of actions for prebiotics in fish.

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To improve the efficacy of the prebiotics and probiotics used for aquaculture, and to screen for more immunostimulatory prebiotics, additional work is needed to characterize the ligand-receptor interactions, signal transduction pathways involved, and the types of cytokines secreted. Investigations into the effects of prebiotics and probiotics on the immune system of fish could further increase their merit if innate immune responses were biologically linked to overall gut health.

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Table 1. The Effects of oral administration of prebiotics on gut immunity. IL, interleukin; COX-2, cyclooxygenase-2; Casp-1, caspase-1; IFN γ , interferon gamma; \rightarrow , no effect; \downarrow , decrease; \uparrow , increase.

Prebiotic	Fish specie	Effect	Reference
Inulin	Gilthead seabream (<i>Sparus aurata</i>)	\uparrow Intraepithelial leucocytes \uparrow IL-8, \rightarrow IL-1, IL-6, Casp-1 and COX-2 genes	Cerezuela et al., 2013a, b
	Atlantic salmon (<i>Salmo salar</i>)	\uparrow Leucocyte infiltration	Bakke- McKellep et al., 2007
MOS	Atlantic cod (<i>Gadus morhua</i>)	\uparrow IL-1 β , IL-8, IL-10, IFN γ genes after challenge	Lokesh et al., 2012
	European sea bass (<i>Dicentrarchus labrax</i>)	\uparrow Lymphocyte and eosinophils \uparrow Lysozyme and bactericidal activity in the mucus \uparrow Prostaglandin production	Torrecillas et al., 2011, 2013

Table 2. The effects of mannanoligosaccharide (MOS) on the humoral immunity of fish

Prebiotic	Doses and administration duration	Fish species	Results	References
MOS	2 g kg ⁻¹ – 90 days	Rainbow trout (30 g)	Antibody titre↑ lysozyme activity↑ ACPA↑ CCPA↑	Staykov <i>et al.</i> (2007)
	2, 4 g kg ⁻¹ – 67 days	European sea bass (<i>Dicentrarchus labrax</i>) (35 g)	lysozyme activity→	Torrecillas <i>et al.</i> (2007)
	2 , 4, 6 g kg ⁻¹ – 60 days	European sea bass (60.64g)	lysozyme activity→	Torrecillas <i>et al.</i> (2011)
	2 g kg ⁻¹ – 4, 6 weeks	Channel catfish (<i>Ictalurus punctatus</i>) (approximately 10.6g)	lysozyme activity→ SH50→ Antibody titre→	Welker <i>et al.</i> (2007)
	2 g kg ⁻¹ – 4 or 5 weeks	Channel catfish (39.1 g)	lysozyme activity→ SH50→ Antibody titre↑	Welker <i>et al.</i> (2012)
	10 g kg ⁻¹ – 45 days	African catfish (<i>Clarias gariepinus</i>) (20 g)	lysozyme activity↑	Yoshida <i>et al.</i> (1995)
	2 g kg ⁻¹ – 12 weeks	snakehead (<i>Channa striata</i>) (10 g)	lysozyme activity↑	Talpur <i>et al.</i> (2014)
	10 g kg ⁻¹ – 8 weeks	red drum (<i>Sciaenops ocellatus</i>) (7 g)	lysozyme activity↑	Zhou <i>et al.</i> (2010)
	10 g kg ⁻¹ – 6 weeks	red drum (10.9 g)	lysozyme activity↑	Buentello <i>et al.</i> (2010)
	10 g kg ⁻¹ – 4 months	Atlantic salmon (<i>Salmo salar</i>) (200.2 g)	lysozyme activity↓	Grisdale-Helland <i>et al.</i> (2008)
	5 g kg ⁻¹ – 8 weeks	Japanese flounder (<i>Paralichthys olivaceus</i>) (21 g)	lysozyme activity→	Ye <i>et al.</i> (2011)

ACPA, the alternative complement pathway activity;

CCPA, the classical complement pathway activity;

SH50, Spontaneous hemolytic complement;

Table 3. The effects of fructooligosaccharide (FOS) on the humoral immunity of fish

Prebiotic	Doses and administration duration	Fish species	Results	References
FOS	3 and 6 g kg ⁻¹ – 56 days	Triangular bream (<i>Megalobrama terminalis</i>) (30.5 g)	ACP, lysozyme, ACH50 activity→ IgM content in 0.3% FOS↑ AKP and PO activities↑	Zhang et al. (2013)
	2 and 4 g kg ⁻¹ – 56 days	Ovate pompano, <i>Trachinotus ovatus</i> (10.32 g)	ACPA→ lysozyme in 0.2% FOS group↑	Zhang et al. (2014)
	10, 20 and 30 g kg ⁻¹ – 7 weeks	Caspian roach (<i>Rutilus rutilus</i>) (0.67 g)	Ig levels ↑ lysozyme activity ↑ ACH50 ↑	Soleimani et al. (2012)
	10 and 20 g kg ⁻¹ – 11 weeks	Stellate sturgeon (<i>Acipenser stellatus</i>) (30.16 g)	lysozyme activity ↑	Akrami et al. (2013)
	2 and 4 g kg ⁻¹ – 10 weeks	Large yellow croaker (<i>Larimichthys crocea</i>) (7.82 g)	lysozyme activity → ACH50 →	Ai et al. (2011)
	5 g kg ⁻¹ – 8 weeks	Japanese flounder (<i>Paralichthys olivaceus</i>) (21 g)	lysozyme activity→	Ye et al. (2011)
	10 g kg ⁻¹ – 8 weeks	Red drum (<i>Sciaenops ocellatus</i>) (7 g)	lysozyme activity↑	Zhou et al. (2010)
	10 g kg ⁻¹ – 6 weeks	red drum (10.9 g)	lysozyme activity↑	Buentello et al. (2010)

Table 4. The effects of Inulin on the humoral immunity of fish

Prebiotic	Doses and administration duration	Fish species	Results	References
Inulin	5 g kg ⁻¹ 2 months	Nile tilapia (<i>Oreochromis niloticus</i>) (11 g)	Lysozyme activity↑	Ibrahim et al. (2010)
	5 g kg ⁻¹ 15 days	hybrid surubim (<i>Pseudoplatystoma</i> sp) (73.6 g)	Lysozyme activity→ Total immunoglobulin↑	Mouriño et al. (2012)
	5 g kg ⁻¹ 10 g kg ⁻¹ 2 weeks	gilthead seabream (<i>Sparus aurata</i> L.) (175 g)	Complement activity→ Peroxidase activity→	Cerezuela et al. (2008)
	10 g kg ⁻¹ 2 and 4 weeks	gilthead seabream (100 g)	Complement activity→ Peroxidase activity→	Cerezuela et al. (2012a)
	10 g kg ⁻¹ 2 weeks	gilthead seabream (50 g)	Complement activity↑ IgM level↑	Cerezuela et al. (2012b)
	10 g kg ⁻¹ 8 weeks	leopard grouper (<i>Mycteroperca rosacea</i>) (35 g)	Lysozyme activity↑ IgM level↑ Myeloperoxidase activity↑	Reyes-Becerril et al. (2014)

Table 5. The effects of other prebiotics on the humoral immunity of fish

Prebiotics	Doses and administration duration	Fish species	Results	References
Galactooligosaccharides (GOS)	10 g kg ⁻¹ –8 weeks	red drum (<i>Sciaenops ocellatus</i>) (7 g)	lysozyme activity↑	Zhou et al. (2010)
	10 g kg ⁻¹ –4 months	Atlantic salmon (<i>Salmo salar</i>) (200.2 g)	lysozyme activity↓	Grisdale-Helland et al. (2008)
Arabinooxylooligosaccharides (AXOS)	AXOS-32-0.30, AXOS-3-0.25, 20 g kg ⁻¹ –12 weeks	Siberian sturgeon (<i>Acipenser baerii</i>) (25.9 g)	ACH50↑ Serum peroxidase↑ lysozyme activity→	Geraylou et al. (2012)
	AXOS-32-0.30, 20 g kg ⁻¹ –4 weeks	Siberian sturgeon (48.4g)	ACH50↑ Serum peroxidase↑ total Ig→	Geraylou et al. (2013)
Levan	1, 2, 5 and 10 g kg ⁻¹ –75 days	Common carp (<i>Cyprinus carpio</i>) (9 g)	lysozyme activity (5 and 10 g kg ⁻¹) ↑	Rairakhwada et al. (2007)
	2.5, 5, 7.5 and 12.5 g kg ⁻¹ –75 days	Carp (<i>Labeo rohita</i>) (4.5g)	lysozyme activity↑	Gupta et al. (2008)
	5, 10, 25 and 50 g kg ⁻¹ –12 weeks	Orange-spotted grouper (<i>Epinephelus coioides</i> H.) (6 g)	lysozyme activity (25 and 50 g kg ⁻¹)↑	Huang et al. (2014)
	2.5, 5 and 7.5 kg ⁻¹ –45 days	Common carp fry (3.26 g)	lysozyme activity (7.5 g kg ⁻¹)↑	Gupta et al. (2014)
Chitooligosaccharide (COS)	2 g kg ⁻¹ –8 weeks	koi (<i>Cyprinus carpio koi</i>) (24.9 g)	lysozyme activity↑ SOD↑	Lin e al. (2012)

Table 6. The effects of commercial prebiotics on different fish species immune response

Products	Doses and administration duration	Fish species	Results	References
Yeast cell wall (Lesaffre Feed Additives)	0.25, 0.5, 1.0, 2.0 and 20 g kg ⁻¹ –72 days	Japanese seabass (<i>Lateolabrax japonicus</i>) (18.3 g)	C3 level→ IgM level→	Yu et al. (2014)
Yeast cell wall (Immunogen [®])	2 g kg ⁻¹ –45 days	Rainbow trout (81.56)	Lysozyme activity↑ Complement activity↑	YarAhmadi et al. (2014a)
Vitacel [®] (fermentable fibr)	10 g kg ⁻¹ –45 days	Rainbow trout (81.56)	Lysozyme activity↑ Complement activity↑	YarAhmadi et al. (2014b)
Yeast cell wall (Immunoster [™])	10, 30 g kg ⁻¹ –8 weeks	Great Sturgeon (<i>Huso huso</i>) (95.68 g)	IgM level→ Lysozyme activity→	Ta'ati et al. (2011)
Brewers yeast (Brewtech [®])	10, 20 g and 40 g kg ⁻¹ –8 weeks	Hybrid striped bass (<i>Morone chrysops</i> × <i>M. saxatilis</i>) (9.7 g)	Lysozyme activity→	Li and Gatlin (2003)
	10, 20 g kg ⁻¹ –4 weeks	Hybrid striped bass (19.7 g)	Lysozyme activity→	Li and Gatlin (2004)
	10, 20 g kg ⁻¹ –16 weeks	Hybrid striped bass (64.5 g, 118 g)	Serum peroxidase↑ lysozyme activity→	Li and Gatlin (2005)
Grobiotic [®] -A	10, 20 g kg ⁻¹ –4 weeks	Hybrid striped bass (19.7 g)	Lysozyme activity→	Li and Gatlin (2004)
	20 g kg ⁻¹ –16 weeks	Hybrid striped bass (64.5 g, 118 g)	Serum peroxidase→ Lysozyme activity→	Li and Gatlin (2005)
	2 g kg ⁻¹ –10 weeks	Golden shiners (<i>Notemigonus crysoleucas</i>) (0.46 g)	ACH50→	Lochmann et al. (2009)