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The use of probiotic bacteria against *Aeromonas* infections in salmonid aquaculture

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A B S T R A C T

Aeromonas species are ubiquitous bacteria in terrestrial and aquatic milieus. In salmonids, they are renowned as enteric pathogens causing haemorrhagic septicaemia, fin rot, soft tissue rot and furunculosis resulting in major die-offs and fish kills. In recent years, there has been a growing interest in controlling disease problems through alternative methods since the use of chemotherapeutic agents may lead to occurrence of resistant bacteria. Lactic acid bacteria may provide protection to create a hostile environment for pathogens. This review summarizes the current understanding of *Aeromonas* infection in salmonids and the use of probiotics in aquaculture for the purpose to prevent these pathogenic bacteria, including the definition and mechanism of probiotics action, and describes their application, prospects and difficulties associated with their use in aquaculture.

Contents

1. Introduction

Global fish production continues to outpace world population growth, and aquaculture remains one of the fastest-growing food producing sectors. In 2012, aquaculture set another all-time production high and now provides almost half of all fish for human food (FAO, 2014). Continuing expansion of aquaculture is viewed as a key strategy to ensure global food and nutrition security and close the “fish-gap”, i.e. the disparity between sea food supply and demand (Ellis et al., 2016). Moreover, under these conditions of intensive production, aquatic species are subjected to high-stress conditions, increasing the incidence of diseases and causing a decrease in productivity (Lara-Flores, 2011; Cruz et al., 2012). Bacterial agents are among the highly encountered causes of diseases in aquaculture and also stressful conditions play important role in establishing and aggravation of the bacterial diseases in fish farms (Saranu et al., 2014; Musefiu and Olasunkanmi, 2015). Diseases caused by *Aeromonas* spp. are commonly implicated in episodes of mortality (Ariole and Oha, 2013).

The genus *Aeromonas* encompasses a diverse group of straight coccobacillary to bacillary Gram-negative bacteria that commonly occur in the aquatic environment and are also isolated from food

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products (Hatje et al., 2014). Initially, aeromonads were recognized only as pathogens that cause systemic illnesses in poikilothermic animals. Today, the genus *Aeromonas* is regarded not only as an important disease-causing pathogen of fish and other cold-blooded species but also as the etiologic agent responsible for a variety of infectious complications in both immunocompetent and immunocompromised persons (Janda and Abbott, 2010).

During the last decades, antibiotics have been used as traditional strategy for fish diseases management (Krishnan, 2014). The public health hazards related to antimicrobial use in aquaculture include the development and spread of antimicrobial resistant bacteria and resistance genes, and the occurrence of antimicrobial residues in products of aquaculture. The greatest potential risk to public health associated with antimicrobial use in aquaculture is thought to be the development of a reservoir of transferable resistance genes in bacteria in aquatic environments from which such genes can be disseminated by horizontal gene transfer to other bacteria and ultimately reach human pathogens (WHO, 2006; Heuer et al., 2009). Considering these factors, as well as the negative effect of residual antibiotics of aquaculture products on human health, the European Union and the USA implemented bans on, or restricted the use of antibiotics. The norms are stringent and there are many events of returning consignments to the exporting countries for not maintaining the prescribed standards (Lara-Flores, 2011).

In addition, the global demand for safe food has prompted the search for natural alternative growth promoters to be used in aquatic feeds. There has been heightened research in developing new dietary supplementation strategies in which various health and growth promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been evaluated (Denev et al., 2009). Hence the use of probiotics i.e., a live microbial feed supplement which benefits the host by modifying the host-associated or ambient microbial community, by enhancing the host response towards disease, by ensuring improved use of feed or enhancing its nutritional value, or by improving the quality of its ambient environment, in aquaculture is being encouraged (Panigrahia et al., 2004). Sugita et al. (2002) suggested that about 1–10% of intestinal bacteria isolated from both marine and freshwater fish exhibit antibacterial activity against fish pathogenic bacteria and can play a role in probiotic treatment of fish. The dietary introduction of probiotic bacteria could reduce mortality of fish challenged with a virulent *Aeromonas* sp. (Cruz et al., 2012). The purpose of this study was to summarize a potential of using probiotic bacteria in the prevention of *Aeromonas* infections in salmonid aquaculture.

2. Characteristics of *Aeromonas* spp.

The taxonomy of the genus *Aeromonas* has been dogged by confusion and controversy (Saavedra et al., 2006). In Bergey's Manual of Systematic Bacteriology the genus was divided into three mesophilic and motile species (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii* biovar *sobria*) and the psychrophilic non-motile species (*Aeromonas salmonicida* subsp. *salmonicida*, *Aeromonas salmonicida* subsp. *masoucida* and *Aeromonas salmonicida* subsp. *smithia*) (Yánez et al., 2003). The psychrophilic species grow best at temperatures between 22 °C and 28 °C. In contrast, mesophilic strains grow optimally between 35 °C and 37 °C, although many strains can also grow at 2–41 °C. Motility of the mesophilic species is facilitated through presence of a single polar flagellum (Percival et al., 2014).

The phylogenetic position of *Aeromonas,* as determined by 16S rRNA gene sequences analysis, is in the class *Gammaproteobacteria*, order *Aeromonadales*, and the family Aeromonadaceae (Tomás, 2012; PHE, 2015). The aeromonads are Gram-negative, rod-shaped, non-spore forming bacteria, facultative anaerobic, catalase and oxidase positive, as well as chemoorganotrophic. They produce diverse kinds of extracellular hydrolytic enzymes such as arylamidases, esterases, amylase, elastase, deoxyribonuclease, chitinase, peptidases, and lipase. Their optimum pH range is between 5.5 and 9 and optimum sodium chloride concentration range is 0–4% (Igbinosa et al., 2012; PHE, 2015).

2.1. Aeromonas Salmonicida

The bacterium *Aeromonas salmonicida* subsp. *salmonicida* is the causative agent of classical furunculosis, a systemic disease of salmonid fish (salmon, trout, etc.) characterized by high morbidity and mortality (Dallaire-Dufresne et al., 2014). *Aeromonas salmonicida* subsp. *salmonicida* is often referred to as typical *Aeromonas salmonicida*, whereas the other strains have been referred to as atypical (Gudmundsdóttir and Björnsdóttir, 2007). Typical and atypical *A. salmonicida* infections have been reported worldwide, with the exception of New Zealand and South America (Hirvelä-Koski, 2005). Australia has managed to keep free of the disease thanks to strict import regulations. In Denmark furunculosis is probably the most important bacterial pathogen in marine rainbow trout farming, while it is less important in freshwater (Buchmann et al., 2012).

Typical furunculosis affects both salmonids and non-salmonid fish and although the route of entry of this bacterium is still debated, there are some reports describing intestinal presence (Bøgwald and Dalmo, 2014). Any age salmonid is susceptible. It has also become a serious problem in marine fish, especially Atlantic salmon culture (Noga, 2010). Furunculosis is a complex disease that takes different forms depending on the health, age, and species of fish as well as the environmental conditions, especially temperature (Dallaire-Dufresne et al., 2014). Furunculosis derives its name from the lesions resembling boils, i.e. furuncles that develop on the skin and musculature of fish (affected by the sub-acute or chronic form of the disease) (Austin and Austin, 2012). It may occur in a peracute, acute, subacute or chronic form, but the distinction and transition between the different forms is not sharp (Buchmann et al., 2012). Peracute disease, which is the least common presentation, has been seen in salmonid fry (Noga, 2010). Furuncles develop from localization of haematogenous bacteria in the muscle or skin, not from an external skin infection (Noga, 2010). These furuncles may rupture exposing open deep ulcers on the surface. A large number of bacteria are released from these lesions and contribute to the spread of the infection (Bruno et al., 2013). During outbreaks, all moribund fish, especially those with skin ulcers, should be promptly removed and disposed of properly (i.e., do not allow contagion to reenter the system). Mortalities are usually low (Noga, 2010). Some fish may recover from disease but the damaged muscle tissue is replaced by scar tissue and the quality of such fish is poor (Buchmann et al., 2012).

Atypical furunculosis forms a very heterogeneous group of bacteria affecting both non-salmonids as well as salmonids. Examples include *A. salmonicida* subsp. *achromogenes*, subsp. *masoucida*, subsp. *pectinolytica* and subsp. *smithia* (Bøgwald and Dalmo, 2014). Atypical *A. salmonicida* display high diversity in biochemical and physiological characters (Buchmann et al., 2012).

Atypical *A. salmonicida* infections associated with disease outbreaks in fish can be manifested, similar to furunculosis. The course of the disease can be peracute, acute, subacute or chronic as described for classical furunculosis. Bacterial isolation should be done from recently developed skin ulcerations as well as from internal organs (Gudmundsdóttir, 1998).

2.2. Aeromonas Hydrophila

Motile aeromonad infection (MAI) is probably the most common bacterial disease of freshwater fish. All freshwater fish are probably susceptible. MAI has been associated with several members of the genus *Aeromonas*, which are ubiquitous in freshwater environments. By far the most important fish pathogen is *A. hydrophila* (syn. *A. liquefaciens*, *A. formicans*), and members of this group are often referred to as the *A.hydrophila* complex. Many other *Aeromonas* species have been taxonomically identified, but only a few aeromonads have been considered as fish pathogens (e.g., *Aeromonas allosaccharophila*, *A. sobria*, *A. jandaei*, *A. bestiarum*, *A. caviae* and *A. veronii*) (Noga, 2010). A new variant *A. hydrophila* subsp. *dhakenis*, which was originally recovered from children with diarrhoea in Bangladesh was determined to be pathogenic to rainbow trout (Orozova et al., 2009). Motile aeromonads are also commonly isolated from the mucosal surfaces and internal organs of clinically healthy fish. Highest prevalence is in organically polluted waters. Ingestion of contaminated feed may also be a source of infection (Noga, 2010).

Clinical signs of motile aeromonad infection range from superficial to deep skin lesions, to a typical, Gram-negative bacterial septicaemia, with or without skin lesions (Cipriano, 2001). The disease is often associated with serious damage and economic losses in rainbow trout (*Oncorhynchus mykiss*) farming industry (Cagatay and Şen, 2014).

Disease is usually precipitated by stress factors like poor water quality, low oxygen tension, high water temperatures, rapid changes in water temperature, high organic loads, concurrent fungal or parasitic infections, transfer of fish, and rough handling. The bacteria are likely proliferate in the intestine or penetrate through skin lesions (Buchmann et al., 2012). Diseased fish transmit the infection horizontally through the water (Bruno et al., 2013).

3. Definition of probiotics

The name probiotic comes from the Greek "pro bios" which means "for life" (Soccol et al., 2010). The term "probiotic" was first used by Lilly and Stillwell (1965) to describe the "substances secreted by one microorganism that stimulate the growth of another." The definition of the term has changed through the years, but perhaps the most appropriate definition was published by an expert consultation at a meeting convened by the FAO and the WHO in October 2001, which states, "probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Determination of definition have had importance even for agriculture, food industry, public health, medicine and microbiology (Kuchta and Pružinec, 2006).

The concept for aquatic probiotics is relatively new. When looking at probiotics intended for an aquatic usage it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics (Denev et al., 2009). The nature of the aquatic species and their intimate interaction with environment forced to a more complicated and precise definition for probiotics, in aquatic hosts, there is no line of demarcation between microbial community inside and outside the host, this is because of the constant interaction with the ecosystem and the host functions. In aquatic environments, the probiotics must be defined to cope with the nature of this sector (Ibrahem, 2015). In the field of aquaculture, Verschuere et al. (2000) extended the concept of probiotic as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment."

As for the aquatic animals such as fish and shrimps, the colonization of the gastrointestinal tract starts immediately after hatching and is completed within a few hours to modulate expression of genes in the digestive tract, thus creating a favourable habitat for them and preventing invasion by other bacteria introduced later into the ecosystem (Carvalho et al., 2012).

Most research has focused on the use of single cultures, and it is largely speculative whether two or even multiple combinations of probiotics are beneficial (Newaj-Fyzul et al., 2014). The approach should be systemic, i.e., based on a mixture of versatile strains capable of acting and interacting under a variety of conditions and able to maintain themselves in a dynamic way. In addition, as has been argued above that in aquaculture the microbial habitat undergoes continuous alterations, allowing constant changes in the structural composition and the functions of the microbial community (Verschuere et al., 2000). Therefore, through the combined application of multiple favourable probiotic candidates, it may be possible to produce greater benefits in aquaculture than the application of single probionts (Nwogu et al., 2011; Ibrahem, 2015).

3.1. Mechanisms of probiotics action

The specific mode of action resulting in the observed host benefits is often difficult to elucidate conclusively, due to the wide range of possible modes of action and the complicated synergistic multi-factorial relationships between them (Merrifield et al., 2010). Several studies on probiotics have been published during the last decade. However, the methodological and ethical limitations of animal studies make it difficult to understand the mechanisms of action of probiotics, and only partial explanations are available (Balcázar et al., 2006). Nevertheless, some possible benefits linked to the administering of probiotics have already been suggested as: production of inhibitory compounds (Vine et al., 2006; Panidyan, 2013; Sopková et al., 2016), competition for chemicals or available energy (Sihag and Sharma, 2012; Ibrahem, 2015), competition for adhesion sites (Mohapatra et al., 2013), inhibition of virulence gene expression or disruption of quorum sensing (Defoirdt et al., 2004; Fuente et al., 2015), improvement of water quality (Cruz et al., 2012; Raja et al., 2015), enhancement of the immune response (Nayak, 2010; Magnadottir, 2010; Hemaiswarya et al., 2013), source of macro and/or micronutrients and enzymatic contribution to digestion (Tuan et al., 2013; Saranu et al., 2014; Andrejčáková et al., 2015).

The mechanisms by which probiotics exert biological effects are still poorly understood (Soccol et al., 2010), therefore there are studies investigate a wide range of probiotics groups in salmonids, such as lactic acid bacteria (*Lactobacillus* spp., *Carnobacterium* spp.) or genus *Vibrio*, *Bacillus* and *Aeromonas*. For a summary of probiotic mechanisms against *Aeromonas* infections in salmonids refer to Table 1.

Different microorganisms may release chemical substances that have a bactericidal or bacteriostatic effect on other microbial populations, which in turn influences the competition for chemicals or available energy (Tinh et al., 2008a). In study of Kim and Austin (2008) both cultures, *Carnobacterium maltaromaticum* (B26) and *Carnobacterium divergens* (B33), isolated from rainbow trout intestine, produced antibacterial substances against *Aeromonas salmonicida*, *Aeromonas hydrophila* and other pathogens. This suggests that the isolate may produce more than one inhibitory compound. Most other studies have also demonstrated that many isolates of carnobacteria have the ability to delay or inhibit growth of *Aeromonas salmonicida*. Of note, Jöborn et al. (1997) demonstrated that *Carnobacterium* K1 inhibited the growth of *Aeromonas salmonicida* in mucus and faecal extracts of rainbow trout.

Newaj-Fyzul et al. (2007) observed the inhibitory activity of *Bacillus subtilis* AB1 against infection by *Aeromonas*. AB1 stimulated immune parameters, specifically stimulating respiratory burst, serum and gut lysozyme, peroxidase, phagocytic killing, total and a1-antiprotease and lymphocyte populations. *Bacillus subtilis* AB1 was effective as a probiotic at controlling infections by a fish-pathogenic *Aeromonas* sp. in rainbow trout.

Pieters et al. (2008) study is the first demonstration in fish of protection by in-feed probiotics against pathogens other than gut associated or intraperitoneal bacterial infections, and specifically against skin infection, namely fin rot. In terms of proximate mechanisms of action, probiotic *Brochothrix thermosphacta* BA211 stimulated phagocytosis and respiratory burst activity, both of which are normally associated with innate antibacterial activity in fish. The further work is clearly needed to assess by what route probiotics stimulate the immune system when administered as a feed supplement. Both probiotics BA211 and GC2 were beneficial to rainbow trout when administered as in-feed supplements against the epidermal bacterial infection fin rot caused by *Aeromonas bestiarum*.

Table 1
Probiotics used against *Aeromonas* infections in salmonid aquaculture.

Identity of the probiotic	Species/method of application	Effects	Reference(s)
Aeromonas hydrophila A3–51	Rainbow trout (*Onchorhynchus mykiss*)/premix with feed	Reduction of infections by *Aeromonas salmonicida*	Irianto and Austin, 2002
Aeromonas media A199	In vitro	Inhibition activity against *A. salmonicida*	Lategan et al., 2006
Aeromonas sobria GC2	Rainbow trout (*Onchorhynchus mykiss*)/feed	Protection against *Aeromonas bestiarum*	Pieters et al., 2008
Aeromonas sobria GC2 and *Bacillus* sp. JB-1	Rainbow trout (*Onchorhynchus mykiss*)/feed	Reduction of mortalities caused by *A. salmonicida*	Brunt et al., 2007
Bacillus subtilis AB1	Rainbow trout (*Onchorhynchus mykiss*)/feed	Controlling infections by a fish-pathogenic *Aeromonas* sp.	Newaj-Fyzul et al., 2007
Brochothrix thermosphacta BA211	Rainbow trout (*Onchorhynchus mykiss*)/feed	Protection against *Aeromonas bestiarum*	Pieters et al., 2008
Carnobacterium sp. (strain K1)	Atlantic salmon (*Salmo salar* L.), rainbow trout (*Onchorhynchus mykiss*)/feed	Inhibition of *A. salmonicida*, reduction of mortality from furunculosis	Robertson et al., 2000
Carnobacterium maltaromaticum B26 *C. divergens* B33	Rainbow trout (*Onchorhynchus mykiss*)/feed	*Aeromonas salmonicida* and *A. hydrophila* inhibition	Kim and Austin, 2008
Enterococcus faecalis and mannan oligosaccharides	Rainbow trout (*Onchorhynchus mykiss*)/feed	Promotion of growth and immune stimulation; decreasing of mortality and frequency of *A. salmonicida*	Rodríguez-Estrada et al., 2013
Lactobacillus delbrueckii subsp. *lactis*	Atlantic salmon (*Salmo salar* L.)/in vitro	Prevention of *Aeromonas salmonicida* subsp. *salmonicida* epithelial damaging effects	Salinas et al., 2008
Lactobacillus rhamnosus ATCC 53103	Rainbow trout (*Onchorhynchus mykiss*)/mixed with feed	Increasing of resistance to *Aeromonas salmonicida* ssp. *salmonicida*; reduction of mortality from furunculosis	Nikoskelainen et al., 2001
Lactococcus lactis ssp. *lactis* CLFP 100 *Lactobacillus sakei* CLFP 202 *and Leuconostoc mesenteroides* CLFP 196	Rainbow trout (*Onchorhynchus mykiss*)/feed	Reduction of mortality from furunculosis	Balcázar et al., 2007a
Lactococcus lactis ssp. *lactis* CLFP 100, *Lactobacillus sakei* CLFP 202 *and Leuconostoc mesenteroides* CLFP 196	Brown trout (*Salmo trutta*)/feed	Modification of the intestinal microbiota and stimulation of the humoral immune response	Balcázar et al., 2007b
Lactococcus lactis ssp. *lactis* CLFP 100, *Leuconostoc mesenteroides* CLFP 196	Brown trout (*Salmo trutta*)/feed	Reduction of mortality from furunculosis	Balcázar et al., 2009
Micrococcus luteus A1–6	Rainbow trout (*Onchorhynchus mykiss*)/premix with feed	Reduction of *Aeromonas salmonicida* infection	Irianto and Austin, 2002
Vibrio fluvialis A3-47S	Rainbow trout (*Onchorhynchus mykiss*)/premix with feed	Controlling *Aeromonas salmonicida* infections	Irianto and Austin, 2002

However, the search for new useful microorganisms continues. There are many studies deal with inhibitory effect of probiotic bacteria against *Aeromonas* infections (Table 1).

The results reported so far with the use of probiotics against *Aeromonas* infections in salmonids are promising. However, in many works the conditions to which the fish are subjected during farming may directly influence the effectiveness of probiotics. Thus, when not subjected to stressful situations, the results often do not show a significant effect of probiotics on the performance of fish. In general, the salmonids from time to time are exposed in unsuitable operating conditions or in conditions of stress during intensive fish farming. These factors, the most commonly include: temperature above or below the thermal comfort range, low levels of dissolved oxygen, presence of other pathogens, poor sanitary conditions, stressful management, change in nutrition, transport, high storage density, sudden change of environment, antibiotic treatment. The effects of adding probiotics should be observed in unsuitable operating conditions or in conditions of stress, when the microflora is unbalanced, primarily in early life stages of salmonids, which are the most sensitive to adverse conditions. Also, the results obtained in experiments with probiotics may be affected by factors such as: type of probiotic microorganism, method and quantity of probiotic administration, additive substances in application form of probiotics, condition of the host, condition of intestinal microbiota, life stage of salmonids, technology of fish farming and aquatic conditions.

3.2. Selection of probiotics

The microorganisms intended for use as probiotics in aquaculture should exert antimicrobial activity and be regarded as safe not only for the aquatic hosts but also for their surrounding environments and humans (Muñoz-Atienza et al., 2013).

A list of characteristics for potential probiotic bacteria to be used in aquaculture species has been reported by Vine et al. (2006) and extended by Merrifield et al. (2010). Among the essential properties to be a probiotic candidate are: being a non-pathogenic microorganism, being free of plasmid-encoded antibiotic resistance genes, and being resistant to bile salts and low pH. Other favourable properties are adequate and rapid growth at host rearing temperature, antagonistic properties against key pathogens, capacity to produce extracellular enzymes that improve feed utilization, viability under normal storage conditions, and acceptable survival under processing conditions (Nates, 2016).

As it is unlikely to find a candidate that will fulfil all of these characteristics, efforts should focus on further exploring the possibilities of simultaneously using several probiotics or synbiotic combination of probiotics and prebiotics (Ibrahem, 2015).

3.3. Safety assessment and regulation of probiotics

Development of probiotics for commercial use in aquaculture is a multidisciplinary process requiring both empirical and fundamental research, full-scale trial and an economic assessment of its uses. Many of the failures in probiotic research can be attributed to the selection of inappropriate microorganisms. Selection steps have been defined, but they need to be adapted to different host species and environments. It is essential to understand the mechanisms of probiotic action and to define selection criteria for potential probiotics. General selection criteria are mainly determined by biosafety (non-pathogenic) considerations, methods of production and processing, method of administration of the probiotic and the location in the body where the microorganisms are expected to be active (Sahu et al., 2008).

Commercial probiotic production should take into account beneficial traits of strain useful during industrial processing. To overcome the problem of inactivation during the manufacturing process, aquaculture industries try to improve the technology by screening for more resistant strains or alternatively by protecting the probiont through microbioencapsulation. By monitoring probiotics and the microbial community structure and dynamics in the manufacture process and in vivo culture system, the viability and effects of probiotics can be documented in detail. For this purpose, nucleic acid-based techniques have been used. Highly discriminative molecular methods such as 16S rRNA gene sequencing and oligonucleotide probes can also be used for accurate probiotic species labelling, which is important for responsible quality control efforts, to build consumer confidence in product labelling, and for safety considerations. The reliable identification of probiotics requires molecular methods with a high taxonomic resolution that are linked to up-to-date identification libraries (Qi et al., 2009).

Quality control of probiotics in aquaculture will become an important issue. With the increased use of molecular methods for the definitive analysis of the bacterial components of probiotic products and for in vivo validation, it is expected that both the probiotics quality and functional properties can be significantly improved. This type of research can aid to the development of adequate technology for the evaluation of the efficiency and safety of microbial agents as probiotics in aquaculture (Denev et al., 2009).

Probiotic organisms for aquaculture need to be first assessed for their innocuousness. Their safety, not only for the host animal but also for users and even consumers of the fish product, needs to be properly documented. Several countries have now developed regulations for the application of microbial feed additives (Lee, 2015).

According to the FAO/WHO (2006), the development of commercial probiotics requires their unequivocal taxonomic identification, as well as their in vitro and in vivo functional characterisation and safety assessment. Significant progress in legislation for the safety evaluation of probiotics, has been made in the USA, Canada, and Europe (EFSA, 2005; HC, 2006; FAO/WHO, 2002); however, no unique standard is available (Gaggía et al., 2010). In the USA, specific utilization of microorganisms for human consumption should possess "GRAS" status ("Generally Recognized As Safe") regulated by the Food and Drug Administration (FDA, 2015). In Europe, the European Food Safety Authority (EFSA) has introduced the concept of Qualified Presumption of Safety (QPS) (EFSA, 2007) similar in purpose to the GRAS approach. The QPS concept provides a generic assessment system for use within EFSA that in principle can be applied to all requests received for the safety assessments of microorganisms deliberately introduced into the food chain. According to recent evaluation, QPS system appears more flexible because it takes into account additional criteria to evaluate the safety of bacterial additives such as a history of safe use in the food industry and the acquisition of antibiotic resistance or virulence determinants. EFSA has published a list of microorganism which possess a known historical safety, proposed for QPS status (Muñoz-Atienza et al., 2013; Otles, 2014; Gupta, 2016). Regulations governing the addition of beneficial microorganisms with probiotic effects to food are less stringent than for feed; the simple fact of belonging to a species with a known safe history of use with a QPS status is enough and no dossier is required (Bernardeau and Vernoux, 2013).

The EU regulations concerning probiotics go back to 1970 with directives No. 70/524/EEC which were amended five times (Ige, 2013). The directives were replaced with new regulation (EC) No. 1831/2003 of the European parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. The regulation set out the rule for its authorization, use, minority, labelling and packaging. In the regulation (EC) No. 1831/2003, the micro-organisms are included in the category "zootechnical additives" and as the functional group within the "gut flora stabilisers" defined as micro-organism or other chemically defined substances, which, when fed to animals, have a positive effect on the gut flora. Requirement for the assessment of microbial feed additives include: 1. Identity, characterisation and conditions of use, method of control; identity of the additive, characterisation of the active agents, characterisation of the additives; physicochemical and technological properties, conditions of use of the additives and control methods. 2. Efficacy, studies on efficacy of probiotics strains must be performed in target species and animal categories. The demonstration for the microbial advantage claim should be based on a minimum of three trials demonstrating a statistically significance ($p < 0.05$) on the specific animal categories.

3.4. Application methods of probiotics in aquaculture

Successful practical application of probiotics is highly dependent on the method of their application and the given dosage, as these are relatively sensitive biological material. It is necessary to choose appropriate dosages in effective forms to obtain optimum probiotic effects. The ability of probiotic microorganisms to survive and multiply in the host strongly influences their effect. Bacteria should be metabolically stable and active in the product and survive during the passage through intestinal tract in large numbers. Probiotic effect of feed may have the desired impact only if they contain at least 10^6 to 10^7 live probiotic bacteria per gram or millilitre (FAO, 2016). The single application should contain maximum recommended dose for that species and should be fed for the total number of days recommended. Feeding lower concentrations of probiotics or decreasing the number of days the probiotic bacteria is fed may cause lower or insufficient colonization of gastrointestinal tract. If this occurs, the probiotic bacteria will not likely be able to control infections that may occur at a fish farm or hatchery.

Probiotics are marketed in two forms a) Dry forms: the dry probiotics that come in packets can be given with feed or applied to water and have to be brewed at a farm site before application. Each kit of dry probiotics contains a packet of dry powder and a packet of enzyme catalyst. Brewing has to be done in a clean disinfected water after emptying the packets and blending thoroughly. Usually, it is brewed at 27–32 °C for 16 to 18 h with continuous aeration. The finished products must be used within 72 h. Maximum aeration is required in semi-intensive culture ponds. If aeration is less, the application of probiotics has to be spread for two consecutive days, applying 50% of the dose each time. b) Liquid forms: The hatcheries generally use liquid forms which are live and ready to act. These liquid forms are directly added to hatchery tanks or blended with farm feed. The liquid forms can be applied any time of the day in indoor hatchery tanks, while it should be applied either in the morning or in the evening in outdoor tanks. Liquid forms give positive results in lesser time when compared to the dry and spore form bacteria, though they are lower in density (Sahu et al., 2008; Cruz et al., 2012). There are no reports of any harmful effect of probiotics but it is found that the biological oxygen demand level may be temporarily increased on its application; therefore it is advisable to provide subsurface aeration to expedite the establishment of probiotic organisms. A minimum dissolved oxygen level of 3% is recommended during probiotic treatment (Denev et al., 2009; Santhanam et al., 2015).

In aquaculture, probiotics can also be encapsulated in feed or live feed like rotifers and *Artemia parthenogenetica*. Bioencapsulation is another efficient application of probiotics to aquatic animals (Tinh et al., 2008b; Akbar et al., 2014). Bioencapsulation in aquaculture can be defined as a process where a live organism incorporates a certain product orally and it becomes a live capsule. The nature of the product given can vary depending on the desire of the aquaculturist. The process is generally directed to the incorporation of some essential elements for the diet (Akbar et al., 2014). Gatesoupe (1991) observed that cultivating rotifers with *Lactobacillus plantarum* significantly increased the population density of rotifers. The amount of aerobic bacteria in rotifer cultures was significantly decreased with L. *plantarum* and the growth of *Aeromonas salmonicida* in rotifers was particularly inhibited by L. *plantarum*.

There have been numerous investigations evaluating the feasibility of supplementing diets with different probiotic bacteria in salmonid species (Gisbert et al., 2013). Currently, there are commercial probiotic products prepared from various bacterial species such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Carnobacterium* sp., and the yeast

Saccharomyces cerevisiae among others, and their use is regulated by careful management recommendations (Cruz et al., 2012). So far only one probiotic was authorized for the use in aquaculture in the European Union, namely *Pediococcus acidilactici* CNCM MA 18/5 M, a member of the lactic acid group bacteria (Regulation (EC) 911/2009; Ramos et al., 2013). Bactocell® Aquaculture (Lallemand Inc., Canada) therefore becomes the first probiotic authorized for such use in aquaculture in the European Union. This authorization is based on the recognition of the quality and safety (QPS status) as well as the efficacy of Bactocell® in beneficially enhancing salmonid and shrimp production (EFSA, 2009). Bactocell® is a zootechnical feed additive based on viable cells of a strain of *Pediococcus acidilactici*. Bactocell® marketed for premixes and pelleted feeds. This product is already authorized for several species including salmonids (EFSA, 2012). In salmonids, Bactocell® is able to improve the quality of the final fish products by increasing the number of well-conformed fish (prevention of Vertebral Compression Syndrome, VCS). This syndrome, which is thought to affect over 20% of rainbow trout harvested constitutes an important economic loss for fish farmers. The use of Bactocell® in the prevention of VCS in salmonids is the subject of an international patent filed by IFREMER and INRA in 2006. In shrimps, Bactocell® is able to increase survival and growth performance. The exploratory trials on the possible use of Bactocell® in aquaculture started in 2002, with first, feasibility trials on live preys (Gatesoupe, 2002). This was followed by numerous field trials and in-depth studies, on shrimps, salmonids and other marine fishes, some of which will be subject to the EU authorization following steeply from this first. The application dossiers for the use of Bactocell® in shrimps and salmonids are therefore the fruit of several years of intellectual investment as well as, research and development conducted in close partnership with renowned researchers, institutions and leading private companies in aquaculture (Bactocell, http://www.aquafeed.com/buyers-guide/suppliers-news-article/2934/Bactocell-the-first-probiotic-authorized-for-use-in-aquaculture-in-the-European-Union/).

The European Commission has approved the use of a preparation of *Bacillus subtilis* (C-3102) (DSM 15544) as a feed additive for laying hens and ornamental fish (Regulation (EU) 2016/897). The additive Calsporin® is a preparation containing viable spores of a single strain of *Bacillus subtilis* C-3102 intended for use as a zootechnical additive (gut flora stabiliser) in feed for ornamental fish and chickens for fattening and rearing, turkeys, piglets, and minor avian species to point of lay. This species is considered by the EFSA to be suitable for the qualified presumption of safety approach to establishing safety for target species, consumers and the environment. The additive was found to meet the criteria for this approach in the context of previous opinions and, as a result, is presumed safe for all target species, including ornamental fish. The use of the additive with feed for ornamental fish is considered unlikely to introduce hazards for users of the product not already considered in previous assessments. The minimum dose proposed for use in feed for ornamental fish is 1×10^{10} colony-forming units (CFU)/kg of complete feedingstuff (EFSA, 2015).

In the last 10 years, in China has been an exponentially growing application of probiotics in aquaculture: at present more than hundred companies are producing many types of probiotics for aquaculture, and probably over 50,000 t of commercial probiotic products are sold annually with a market value estimated at 50 million euros. The probiotics used in Chinese aquaculture are mainly photosynthetic bacteria (*Rhodopseudomonas palustris*, *Rubrivivax gelatinosa*, *Rhodobacter capsulata*, *R. sphaeroides*, *Phaeospirillum fulvum*, etc.), antagonistic bacteria (*Pseudoalteromonas* sp., *Flavobacterium* sp., *Alteromonas* sp., *Phaeobacter* sp., *Bacillus* sp., etc.), microorganisms for nutritional and enzymatic contribution to the digestion (lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium*, yeasts, etc.), bacteria for improving water quality (nitrifying bacteria, denitrifiers, etc.), *Bdellovibrio*, and other probiotics. Recently, an integrated approach by using combined probiotics (= microecologics) is gaining popularity. Currently, the most popular commercial probiotic in China is Effective Microorganisms (EM) from Dr. Teruo Higa's EM Technology, Japan. EM consists of a group of beneficial and non-pathogenic microorganisms, such as lactic acid bacteria, photosynthetic bacteria, yeasts and *Actinomyces*. Overall, the research of probiotics in aquaculture of China is still in its early stage, and not much of commercial probiotics products were licensed in China so far (Qi et al., 2009).

Under experimental conditions, some probiotics that are used and documented for human application have been utilised as well in aquaculture. LEVUCELL® SB is a natural and well documented live yeast *Saccharomyces cerevisiae boulardii* (CNCM I-1079 Pasteur institute). LEVUCELL® SB allows optimal intestinal balance which positively impacts and performance, validated by numerous scientific and farm trials. LEVUCELL® SB for aquaculture is only available outside EU (Maricchiolo et al., 2015; Navarrete and Tovar-Ramírez, 2014).

Adel et al. (2016) have examined the use of Aqualase®, a yeast-based commercial probiotic in the production of rainbow trout as a way to improve fish health and production. Aqualase® predominantly composed of two yeast species *Sacchcaromyces cerevisiae* and *Sacchcaromyces elipsoedas*. The results of this study revealed that the commercial probotic mixture Aqualase® (Thepax®) incorporated in the diet could consequently improve health and performance of the cultured fish. The benefits from this yeast-based probiotic include the modulation of intestinal microbiota, enhancement of immune responses, particularly the inhibitory potential of skin mucus against fish pathogens, contribution to intestinal enzymatic physiology and last, improvement of growth performance. Aqualase® is only available outside EU.

The above mentioned products are marketed as liquids or powders and in different concentrated forms, to ensure their application at different stages of feed production or their direct administration into the water. It is worth noticing that producers do not state exact strains of bacteria in probiotic preparations in countries having less-stringent regulations (Asia, South and Central America). Only quantity of bacteria is labeled.

4. Conclusion

This review describes negative effect of *Aeromonas* infections in intensive salmonid farming, the need of using probiotics to increase a resistance against these infections and by that increasing aquaculture's productivity. Many in vitro and few in vivo studies confirm promising results of probiotics on a fish immune system, weight gain and superior quality of final aquaculture products. As there is only one probiotic strain approved in EU, there is a need for further research to aquire more appropriate strains to test them in more in vivo studies in unsuitable operating conditions or in conditions of stress. Application form needs to be adjusted to increase probiotics' effect on organism and protect the probiotics itself.

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