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Characterization of Probiotic Bacteria in Fish

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ABSTRACT

The effects of probiotics on the growth performance and other beneficial activities in fish have well been documented. Characterization or screening of probiotic bacteria is the most important stage after bacteria isolation and identification. Generally, important phenotypic criteria for screening of probiotic bacteria are carbohydrate fermentation pattern, resistance to different NaCl and bile salt concentrations. In addition, growth at different temperatures, pH and nutrition media and also antagonistic effect against fish pathogens and antibiotic sensitivity are other criteria. Moreover, genetic techniques play a very important role when characterization of probiotic bacteria is considered. Identification of probiotic LAB with regards to phenotypic and genomic methods has been studied with a good amount of success. The important genera which are mostly selected for probiotic purpose are generally Lactobacilli, Bifidobacteria and Enterococci, but other lactic acid bacteria (LAB) are also known as good probiotics candidates.

Keywords: Probiotic, Characterization, Acid, Bile salt tolerance

INTRODUCTION

The last definition was put forward in 2005, which defined probiotics as "live microbial cultures added to feed or environment (water) to increase viability (survival) of the host". Although it has been reported that the gut microbiota of fish is little different than in homoeothermic animals, the gastrointestinal (GI) tract of fish might not be as simple as believe. The gut microbiota of fish as well as warm-blooded (endothermic) animals classified as autochthonous (able to colonize the epithelial surface of the host gut) and as allochthonous (transient). It means that the varied microbiota of the gut can be divided into two major categories; a) bacteria that can be normally isolated (autochthonous) from the gastrointestinal tract; b) bacteria that are not normally isolated (allochthonous) from the gastrointestinal tract of fish [1,2].

Probiotic bacteria can prevent the growth of harmful bacteria by colonization in the gut and produce organic acids (such as lactic acid and acetic acid) and antimicrobial compounds (such as hydrogen peroxide and ethanol) [3,4]. Moreover, lactic acid bacteria (LAB) which compete with bacterial pathogens for survivability, viability and live in the GI tract can be valuable and valid alternatives to the prophylactic use of antibiotics and biocides [4]. According to many reports, indigenous microbiotas of fish or rearing environment are the best choice as probiotics, because they can naturally inhibit pathogenic bacteria [5-7]. In addition, LABs that are isolated from intestinal origin and dairy products are considered to be the main source of probiotics [3]. Both obligate and facultative anaerobes have been isolated from fish but with much less frequency than from mammals [8,9]. A consequence of the specificity of aquatic microbiota is that the most efficient probiotics for aquaculture may be different from those of terrestrial species [10].

LACTIC ACID BACTERIA (LAB) AS A MAIN GROUP OF PROBIOTIC BACTERIA

The LAB are a group of bacteria which are Gram-positive, rod and coccus-shaped, non-spore forming and non-motile, catalase-negative and oxidase-negative that have some physiological and ecological characteristics in common. They have less than 55% mol G+C content in their DNA [10]. They produce lactic acid as major metabolite during fermentation of carbohydrates [10,11].

New findings on LAB and their properties have led to their

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taxonomy change during the last few years. Up to now, this group comprises the following genera: Lactobacilus, Carnobacterium (both rod), Enterococcus, Aerococcus, Alloicoccus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, Weissella (all cocci) [7,12,13] and also Bifidobacteria [10,11].

Lactic acid bacteria are micro or non-aerobic but are aerotolerant and acid tolerant. These bacteria can be divided into two physiological groups; a) heterofermentative which produce CO_2, lactic acid, acetic acid, ethanol and mannitol from hexose sugars, b) homofermentative which produce primarily lactic acid from hexoses. Traditionally, they have been classified on the basis of phenotypic properties such as mode of glucose fermentation, growth at different temperatures, configuration of LAB produced (L/D) and fermentation of carbohydrates. In general, it is thought that probiotic properties are specific for strains. Thus, the requirement for strain detection and identification are very important [11].

ISOLATION OF SOME LAB FROM AQUATIC ANIMALS

Dyer [14] was the first scientist who isolated Lactobacilli from the skin, gills and GI tract of Atlantic cod fish (*Gadus morhua*). Lactic acid bacteria have also been isolated from terrestrial animals, dairy and fermented products and even soil and plants as well [10,15]. *Lactobacillus delbreuckii* has been isolated from adult European sea bass (*Dicentrarchus labrax* L.) gut and showed positive probiotic effects on European sea bass juveniles [16]. This strain could also modulate the fish microbiota in gut [17]. *Enterococcus fecalis* was isolated from the freshwater fish, Bonga (*Ethmalosa firmbriata*) and from the intestine, gills and skin of both fresh and smoked fish [18]. It has also been reported in tropical cooked, peeled shrimp [19] and in traditionally processed *fish* products [20-26]. In addition, Thapa et al. [27] described both *En. fecalis* and *En. fecium* in smoked and sun-dried fish. Although Enterococci have been reported for prawn, marine and freshwater fishes, only some researchers suggested them to be used as probiotic in human and animals [10]. Moreover, those reports showed Enterococcus as normal microbiota in some aquatic animals. Hovda et al. [23] isolated *Lactobacillus* sp. and *Lactococcus* sp from the stomach and intestine of Atlantic salmon (*Salmo salar* L.). They reported the presence of *Lactobacillus fermentum* in the gut of this fish. *Lactobacillus fermentum* has also been isolated from the intestine of rainbow trout (*Oncorhynchus mykiss*) [21], Atlantic salmon [22] and prawn as well [23]. Researchers reported the presence of *L. fermentum* in the gut of Thai freshwater silver barb fish (*Barbonymus gonionotus*). The presence of Leuconostoc species in the four kinds of fermented fish has been reported [24]. Lyhs [25] also isolated *Leuconostoc mesenteroides* from cold-smoked rainbow trout. Eight strains as genus Leuconostoc from brine shrimp, gravad fish, smoked tuna, salmon, fresh and salted cod were isolated [26]. They also isolated several adherents *Leu. mesenteroides* from the stomach, small and large intestine of Arctic charr, and also this LAB was isolated from smoked and sun-dried fish [27]. Also, *Aerococcus viridans* as a pathogenic bacterium has been isolated from crustaceans, sea turtles, pigs and human infections [28], lobster (*Homarus americanus*) [29]. Salminen [10] suggested that Aerococcus strains have rarely been isolated from aquatic animals and only one study reported the presence of five strains in Atlantic salmon gut.

PROBIOTIC CRITERIA

The important genera which are mostly selected for probiotic purpose are generally Lactobacilli, Bifidobacteria and Enterococci [11], but other LAB is also known as good probiotics candidates. Among the LAB, Lactobacilli is a major group that has acceptable probiotic abilities [10].

The following criteria are recommended when LAB are selected to be used as a main group of probiotic bacteria [7,8,10,30,31]:

1) Adhere to gut cells
2) Reduce pathogen bacteria adherence
3) Compete for essential nutrients
4) Stimulate immune system
5) Persist and multiply
6) Organic acid production, hydrogen peroxide and bacteriocins against pathogen growth
7) Safe and non-invasive and non-pathogenic
8) Aggregate and form a normal balanced flora
9) Indigenous to the environment to which it will be used.

Other criteria such as the enhancing of GI tract morphology, inhibition of virulence gene expression and aiding digestive function have also been suggested [32].

CHARACTERIZATION OF LACTIC ACID BACTERIA

In total, important phenotypic criteria in early stages for screening of probiotic bacteria are carbohydrate fermentation pattern, resistance to different NaCl, pH and bile salt concentrations, growth on different nutrition media, sugar tolerance, growth at different temperatures and antibiotic susceptibility [7,14]. Moreover, other characteristics which included tolerance to ammonia nitrogen, tolerance to simulated human gastrointestinal tract and assay of cholesterol assimilation can be used [33].

Recently, in modern taxonomic phenotypic methods, analyses of cell wall composition (peptidoglycan) and fermentation pathways of pentoses and hexoses are studied. In some industries such as dairy industry, Lactobacillus and

Bifidobacterium are used in developed countries as a significant proportion of probiotic bacteria. Since these two species have been isolated from different parts of the GI tract, the terminal ileum and colon are the preferred sites [11]. The enzyme fructose-6-phosphate phospho-ketolase is known as a key enzyme in the glycolytic pathway. It serves as a taxonomic character in identifying LAB genera but does not enable inter-specific differentiation. However, the careful selection of the strain is very important to ensure that probiotic bacteria do not have any negative effects on the host [11].

Genetic techniques play a very important role when characterization of probiotic bacteria is considered. Identification of probiotic bacteria with regards to phenotypic and genomic methods has been studied with a good amount of success [11]. Since in genetic studies, plasmid profiling showed that extra chromosomal DNA was not stable, techniques based on chromosomal DNA were developed. These methods comprise restriction enzyme analysis, randomly amplified polymorphic and gradient gel electrophoresis. The efficacy of genotypic methods is based on strain identification [11]. It is necessary to use both phenotypic and genotypic techniques for identification of strain in bacteria correctly [9,11].

IMPORTANCE OF ACID AND BILE SALT TOLERANCE

The most important criteria in bacteria characterization are ability to tolerate low pH and bile salt concentrations [14]. Probiotic bacteria should reach the final destination in the gut to exert their beneficial effects on the host [34]. Hence, it is necessary for probiotics to be able to tolerate acid in the stomach and bile salts in the intestine [10,34,35].

Acid and bile salts may have both individual and combined effects. Also, there are variations in the acid and bile salt tolerance among probiotic bacteria [34]. When the LAB or other probiotic bacteria passed the stomach, established and colonized in the fish intestine, they will survive under the stress conditions [11,34]. Some authors believed that the ability to tolerate the presence of pancreatic enzymes can be considered as another criterion for selection of probiotics [3].

Cebeci and Gurakan [34] declared that *L. plantarum* could survive at pH 4 and 0.3% of bile salt. *Lactobacillus plantarum* pH 4 was able to grow at pH between 6 and 10 and bile salt ranging from 0% to 0.4%. Kim and Austin [15] reported the growth of probiotic Carnobacteria strains isolated from the rainbow trout intestine occurred at pH 5-10. Samelis [36] determined that only some *Lactobacilli* sp. isolated from Greek dry salami could grow at pH 3.9. Balcazar [21] investigated growth of isolated LAB (such as *L. fermentum* and *L. plantarum*) from the intestine of rainbow trout at pH 1-6.5 and 2.5-10% extracted bile. The bacteria showed growth at pH 2.5-6.5 and they tolerated bile concentration for 1.5 h and no significant changes in viable counts were observed. Similar results were reported by Badis [36] and Thapa [27].

GROWTH OF LAB AT DIFFERENT NaCl AND TEMPERATURES

The ability of probiotic bacteria (particularly LAB) for growth at different NaCl concentrations and temperatures are also investigated in probiotic characterization [15]. Temperature is an important parameter when it comes to bacterial growth [7]. Nguyen [36] determined the growth of *L. plantarum* pH 4 in NaCl concentrations up to 10%. This bacterium could grow up to 6% concentrations and also showed ability to grow at temperature between 25°C and 45°C when tested at temperatures ranging from 5°C to 60°C, whereas two probiotic Carnobacteria strains isolated from the rainbow trout intestine were able to grow at 0-15% (w/v) NaCl among ranging 0-20% and grew at 10-37°C [14]. Gonzales [13] isolated *Carnobacterium* sp. from brown trout and tested on 4, 7 and 10% NaCl. They found that the isolates did not grow in presence of 8% NaCl. In addition, the growth ability was inhibited at 4°C and 45°C.

The growth of some Lactobacillus bacteria in the presence of 8 and 10% NaCl (w/v) in MRS broth was reported by Samelis et al. [36]. They also observed that these isolates could grow after incubation at 15°C, 37°C and 45°C for 5 days and at 4°C and 10°C for 12 days. Similar results were reported by Badis [36] and Thapa [27]. Lactic acid bacteria species that were isolated from smoked and sun-dried fish tested for the ability to grow in different concentration of NaCl of 6.5, 10 and 18% (w/v) in MRS broth [27].

ANTAGONISTIC EFFECT AGAINST FISH PATHOGEN

The antagonism between microorganisms in nature usually occurs [10]. Lactic acid bacteria are effective probiotics that have inhibitory activities against pathogenic bacteria. They produce antibacterial substances such as bacteriocin, lactic acid, hydrogen peroxide, acetaldehyde and diacetyl to inhibit pathogens activities [8,10,37]. Aly [38] reported that the growth of *A. hydrophila* as fish pathogen was inhibited by three species of *Bacillus* bacteria as probiotic and Rengpipat [39] confirmed growth inhibition on *A. hydrophila*using a cell-free cultured broth of five LAB. Kim and Austin [15] reported the antibacterial ability of two probiotic strains, Carnobacterium *B26* and *B33*, isolated from the rainbow trout intestine against *A. hydrophila* and *A. salmonicida*. These strains inhibited the growth of both *A. hydrophila and A. salmonicida*. Moreover, it was reported by Pan [40] that *L. delbrueckii* had greater inhibition effect than *Clostridium butyricum* against *A. hydrophila* in farmed fish. Vine [41] isolated 106 bacteria from the stomach and intestine of common clown fish (*Amphiprion percula*). The extracellular products of five LABs were able to inhibit the growth of a wide variety of pathogens such as *A. hydrophila, A.*

salmonicida, Vibrio harveyi, V. anguillarum V. damsela and *V. alginolyticus*. The intestinal tract and feces can serve as enrichment sites for pathogenic bacteria such as Aeromonas and Vibrio species and probiotics or intestinal bacteria with antagonistic activity may be used to reduce or inhibit pathogen activities. Moreover, the competitive exclusion can be influenced by the production of inhibitory substances [21].

ANTIBIOTIC SENSITIVITY

Antibiotic-resistant probiotic may be advantageous in the case of administration of antibiotics to the fish and the establishment of the beneficial microorganisms in the intestine for prolonged periods [15]. The resistance of LAB to specific antibiotics indicates that the LAB can be given at the same time when antibiotic treatment is required and also the microbiota of the intestine can recover quickly [15,34]. The antibiotic susceptibility of Carnobacterium *B26* and *B33* strains were tested by Kim and Austin [15]. They reported resistance to ampicillin, gentamycin, kanamycin, streptomycin and penicillin G, but showed sensitivity to chloramphenicol, tetracycline and cotrimaxazole.

CONCLUSION

Finding of probiotic properties (as probiotic bacteria characterization) is the most important stage after bacteria isolation and identification. Assessment of some abilities such as carbohydrate fermentation, resistance to different pH and bile salt and NaCl concentrations, growth at different temperatures, antagonistic effect against fish pathogens and antibiotic susceptibility are the capable factors to use a bacterium as a probiotic.

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