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Evaluation methods for development and selection of novel probiotics

Abstract

Probiotics is currently one of the science–driven products which have undergone considerable evolution with acclaimed health benefit. Besides the discovery of antibiotics some years ago, probiotics has found considerable applications in life sciences, aquaculture, poultry, piggery, animal health, and human healthcare. There are many novel putative probiotic organisms that could be found in different substrates or carbon sources among bacteria, bacteriophages, fungi, yeasts, microalgae etc. A search approach to developing candidate probionts could be made among these variable sources. Invariably, the methods for isolation and evaluation of the probiotic organisms are many depending on the purpose of use. To wit, different methods are used in aquaculture industry or animal health and human healthcare. For instance, the Food and Agriculture organization *FAO* of the United Nation/ World Health organization *UNO/WHO* has already developed international guidelines for the evaluation of probiotics meant for the later. Consequently, this chapter reviews the different methods and approaches for development and evaluation of novel potential probiotics for aquaculture production, taking into considerations the differences in environments and the complex needs and nature of aquatic species. Qualifying a strain of bacterium as a probiotics and selection of such strain for probiotic purposes has not been easy. Principally, scientific driven approaches have been used to primarily decipher the specific trait a desirable probiotic strain should possess, and also developed methods used for selecting and evaluating candidate probiotics. This review will addressed the different methodologies which have been used to analyze microbial cells, which promises to serves for probiotic strains for use in aquaculture industry.

Keywords: probiotics, bacteria, health mechanism, aquatic animals

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Introduction

Probiotic species are numerous so far as their applications are different, especially where probiotics are to be used, including aquaculture sector. Different source of putative probiotics are known and are currently in used.[1,2] And they include bacteria,[3] bacteriophages,[4] yeasts[5] and microalgae.[6]

Often, this calls for the need to specify the methodology used for screening and evaluating potential probiotics for targeted or desired purpose. Accordingly, the methods used for isolating and evaluating probiotics in aquaculture are many, taking into consideration the differences in environments and nutritional needs of host species. Many probiotics have demonstrated good functionality by stimulating the immune system and improving the host resistance to infectious diseases.[7,8] Furthermore, they are known to colonize the gastrointestinal canal, body skin and scales, mucus membrane and linings of the aquatic animals. These pathways may be evaluated directly or indirectly for the present and subsequent isolation of probiotic organisms. Their functionality in the host may also be assessed from their host blood samples after due exposure for specific time duration. The logical supposition here is that any microbionts that can colonize their hosts without causing pathogenic diseases could possibly function as effective probiotics. Thus, microbial isolates of any ecological community isolated from aquatic animals which can establish themselves in the host and without causing disease scenario can function as probiotics of aquaculture. This review discusses the different methods used for selecting and evaluating novel probiotic organisms used in aquaculture. Furthermore, it emphasis some key issues that should be taken into considerations in probiotic development used in aquaculture industry and make some suggestions for future improvement.

A search approach to isolating and screening of putative probiotics

The methods of getting putative probiotics is usually by isolating and screening of microbial organisms from various sources, where they occur as normal commensal and without being virulence. The various sources or the host of the isolates are used to gather important background information about the putative probiotics. In aquaculture, the usual sources where this putative probiotics are isolated from, for analysis and screening is the aquatic animal itself (in this case fish, if the search is to be use for fish). The principal examples are the mucus linings of the fish gastrointestinal tract, the gastrointestinal tract (GIT) of fish,[9] or the fish skin/scales,[10] fish eggs[11] or from the cultured water or environment.[12] From these sites, putative isolates can be obtained and plated out on multipurpose culture medium, such as nutrient agar *NA* or tryptone soya agar *TSA* or on selective media as the case may be and incubated 24 h or more. For example, Imo and Nya[13] made a swapped from the gastrointestinal tract microbiota of African Cat fish and from fermented soybean milk as a potential source for probiotics. In another instances, Boutin et al.,[10] isolated putative probiotics from the skin microbiota of brook charr *Salvelinus fontinalis* for analysis as potential source for probiotics which was effective against *Flavobacterium*.

However, the putative microbial isolates are collected and wash in saline, plated out on multipurpose culture medium and incubated at room temperature for or more than 24 h depending on the growth physiology of the isolates. At this point, they can be sub-cultured and cross-streaked onto appropriate growth medium for further analysis. This is followed by characterization of the isolates, usually using morphological, biochemical and molecular characterization methods leading to rapid identification of the probiotic organisms. The most

common molecular characterization for identification of unknown organisms is the DNA Barcoding Method[14] and Simple Sequence Repeat-SSR marker[15] for sequencing of conserved regions within the genome, such as 16S ribosomal DNA in bacteria and ITS regions for yeasts and fungi.[16] The identification is done by comparing the sequences with data stored in biotechnology data-bank or GenBank and in addition with phylogenetic analysis.

Furthermore, multiple tests may be required to confirm the identity of a microorganism and to give more information of the characteristics of the potential probiotics.[17,18] Sometimes whole genome sequencing (WGS) may be required as it is done by the European Food Safety Authority (EFSA) for microbial isolates intended for use as feed additives.[19]

Antagonistic activity against other pathogens

Customarily, to develop potential probiotics for use in aquaculture, we need to screen microbial isolates for the presence of antagonistic property or anti-pathogenicity against a target pathogens in an *in-vitro* setting.[20] There are several methods available for one to use in going about this:

i. Based on co-culture in the same plate of agar medium and the subsequent observation of inhibitory zone and

ii. Observation of competitive growth among the microorganisms.

For example, using cross-streaking method, overlayings, agar/well diffusion, spot-on-lawns and broth co-culture methods can be used for achieving this purpose.[10,21,22]

Furthermore, it is a well known fact that many microbial isolates can produce extracellular products with antimicrobial property such as enzymes, bacteriocins and other compounds which can inhibit the growth and inactivate pathogenic organisms. On this note, these extracellular products can be evaluated for selection of potential probiotics, by several inhibitory assays methods such as:

i. Agar diffusion or turbidometric assays. This is carry out with a cell-free supernatant from the culture growth medium of probiotic microorganisms in optimal conditions.[23–25] Precautions should be applied when using these methods, as bioactive compounds could be produced by microorganisms at its specific growth phase, especially when there is inadequate availability of certain nutrients. Arguably, bioactivity could be due to a mixture of several compounds. Vine et al.,[26] opined that this method need to be approached systematically to take account of all these variables and suggested that when bioactive compounds with anti-pathogenic activity are found, they could be extracted, and their functional properties studied in more details.[27]

ii. Competitive exclusion: Competitive exclusion of pathogens by probiotics can be achieved through many processes. However, only the ability to produce bioactive compounds is to be taken into account. Principally, many studies have been conducted to find out how fish mucus can influences the competitive growth of probiotic bacterium and pathogenic bacteria[27] and how they competitively attach themselves on fish mucus by radiolabel activity.[28–30]

iii. Adherence property of probiotic and pathogenic bacteria from different intestinal cells segments have also been studied on primary cell cultures. Lazado et al.,[31] reported fewer epithelial cells damaged detected in histological samples of fish gut after being exposed to both pathogenic bacteria and probiotics *in vitro*. Ringø *et al.,*[32] made comparison between pathogenic bacteria and probiotics in histological samples after exposure.

iv. Possession of anti-pathogenic property: Anti-pathogenic activity of the putative probiotic bacterium needs to be tested *in vivo*, since *in vitro* results do not necessarily translate to same activity in animal model.[22,33]

v. Furthermore, anti-pathogenic activity is usually tested by probiotic supplementation on animal model for specific period usually 14 days with subsequent experimental challenge with virulent pathogen and survival observation of the animal for mortality, which also account for or taken for the probiotics ability to protect the animal from infectious pathogens.[9,11,34,35,] However, in doing so, many things should be taken into consideration viz: the weight and age of the experimental animals or target species, challenged dose and the supplementation. The challenged dose need to be determined in tandem with the specific pathogenic strain suspected in the aquatic animal. That is the more reason it is often necessary to calculate the lethal dose of the pathogen for the target aquatic animal, since one dose is not necessarily suitable for all the experimental animals.[9,11] However, during *in vivo* challenges test, the performance of the potential probiotics needs to be compared with appropriate controls, where the virulence of the pathogen may be determined. Furthermore, it is a standard practice to re-isolate the pathogen responsible for killing of the animal and identification of same for similarity checks with the pathogens used in the challenges experiment.

Attachment for colonization

The Attachment processes and subsequent Colonization of the potential probiotics relates to its ability to withstand the horrible gastrointestinal tract GIT environment of the host animal, Furthermore, of utmost important is the establishment in the host cells and its proliferation in the presence of other commensal organisms.

It is a known practice that most of the probiotics used in aquaculture are orally administered and as such they enter the host through the gastrointestinal tract GIT.[20] However, when probiotics are administered in this way, it become critical to withstand the acidic conditions of the GIT environment, which is also crucial for the survival and efficacy of the probiotic organisms.[36]

In screening for putative probiotic organisms, this aspect is often addressed initially in the *in vitro* studies, by addition of crude bile or synthetic gastric juice to the cultured medium where probiotics parameters such as viability and growth of putative probiotics are assessed.[18,37] Furthermore, *in vivo* tests have also been carried out; where numbers of viable cells are assessed after feeding trial with probiotics dietary supplements.[24,37,38] Nevertheless, *in vivo* tests are meant to provide information about the survival, adhesion, proliferation and colonization of the probiotic bacteria.[39] Adhesion and subsequent colonization of probiotics in the GIT mucosal have been demonstrated both *in vitro*[31] and *in vivo* in aquactic animal studies[35,40] For instance, Merriefield et al.,[38] had examined the colonization patterns of probiotics in the GIT using histological samples and reported that the adhesion and colonization properties of the probiotics may be affected by the method of administration. In another instances, Korkea-aho et al.,[11] in their studies reported that the colonization properties of probiotics are seriously affected when adding probiotics through water rather than through feed supplementation. This was in line with what Kewcharoen and Srisapoome[12] reported earlier.

Assessment of immunostimulatory properties of probiotic candidates

Probiotics have been known for the enhancement of humoral and cellular immune responses,[41–43] and improved the host resistance to infectious diseases. Therefore they offer immunostimulation to their host.[8] However, this property is often assessed through the innate immunity which is the primary defense mechanism against pathogens in host animals by using blood samples from the animal after feeding trial with a probiotics diets for a specific duration, usually 14 days. Parameters considered in this assessment are immune cells such as Leukocytes, monocytes, neutrophils and macrophages. Their proliferation showed indication of immunostimulation as a result of probiotic diets. These proliferations of immune cells, as compared with the Control blood cells and may be determined by:

i. Conducting microscopic blood count,

ii. Determining the percentage hematocrit and leukocrit and

iii. Phagocytic activity of neutrophils and macrophages always known to be activated during immunostimulation.

iv. The respiratory burst activity of phagocytosis.

However, head kidney is often used to isolate macrophages for the determination of phagocytosis. Principally, the respiratory burst activity of the phagocytosis is evaluated from reactive oxygen species (ROS) of innate immune cells.

Furthermore, humoral immunity, such as bacteriolytic enzymes, Lysozyme, Anti-protease, interferons and complement activity are also known to be activated during immunostimulation, these humoral components are always assessed from serum of the host animal as follows:

Bacteriolytic enzymes: this parameter is measured by adding serum prepared from aquatic animal fed probiotics to the cultured pathogenic bacteria medium, and assessing the growth of the pathogen and compared to the control animal. Sometime broth culture is used to grow the bacetria.[44]

Lysozyme: It is seen as the first defense mechanism found in the mucus covering the scales, bucal cavity and GIT. It can therefore, be isolated and measured from the mucus of the host animal.[9] Lysozyme is bacteriolytic enzymes, many bacterial cells are sensitive to it. Lysozyme activity can be measured by using *Micrococcus lysodeikticus* or *Micrococcus luteus* cells as an indicator.[24,44]

Antiprotease: This is another bacteriolytic enzyme, but found in fish serum. It serves as inhibitors of proteolytic enzymes secreted by pathogenic bacteria. Total anti-proteases may be measured as α1-antiprotease and α-2 macroglobulin, this is carryout when trypsin is added to fish serum, and antitrypsin activity is observed and measured.[9]

Complement activity: Many pathways are responsible for complement activity including and indeed some antibodies activity, like primary antibody, immunoglobulin M *IgM*. Complement exerts an important role in innate immunity and may be measured from serum of the host animal.[45,46] However, immunoglobulins act against pathogens and thus form the basis of acquired immune defense. The level of this immunoglobulin (immune cells) in the serum may be measured by the enzyme-linked immunosorbent assay ELISA.[33,37]

Interferons (IFNs): Are inflammatory cytokines which secrete signaling molecules and are sometimes activated by leukocytes and other immune cells. They are many interferons or inflammatory cytokines, such as interleukins (ILs), tumor necrosis factors *TNFs, TNFα, IL1β, IL 4, IL6, IL8* and *IFNγ*. Activation and proliferation of these immune-related genes may be measured from gene expression using real-time reverse transcriptase polymerase chain reaction *RT-PCR*.[17,33,47,48] Furthermore, as a rule, this is usually after probiotics exposure for specify duration. Standen et al.,[49] reported that cells obtained from immune-related organs, such as thymus gland, the head kidney and the intestinal epithelial cells of host animal, often expressed immune-related genes. This is why they are always investigated for the regulation of immune-related genes. However, immune regulation activity of probiotics in small aquatic animals like the larvae, fingerlings etc, is often determined by measuring or taking the analysis of immune-related gene expression. Critically, this done by using the whole animals rather than component organs or tissues.[35]

Evaluation of genome sequencing

Probiotic strains have been selected and confirmed through genome sequencing. They have been known to contain no plasmids, antibiotic resistant genes or deleterious genes.[50] However, Robinson and Samona,[51] reported how human clinical study has shown their ability to control microbial populations by competitive exclusion, thus leading to improvement in digestion and maintenance of general health. Experimental search for novel candidate probiotics notably, starts with the isolation of organisms from faecal, mucosal or intestinal samples follow by culturing and subsequently analysing the cultured sample and selection of the desirable strains- bacteria, algae or yeast that has been characterized and found to be of health benefits and non-virulence. However, this approach has major shortcomings, as it is recognized that many microbes cannot be cultivated by standard culture techniques.[52] Nevertheless, culture techniques are still very powerful and essential to obtaining a complete picture of the microbial diversity in the intestinal environment. Principally, to study such a complex environment, the combination of both culture and non-culture; biochemical and molecular based techniques are required.[53] This translates to the fact that there are *in vitro* microbiological, biochemical methods, and molecular methods including immunological techniques[54] available for use as explain earlier in the text. Furthermore, the evaluation of genome sequencing leads to identification and characterization of the candidate isolates by using molecular tools that has the ability to identify isolates and also decipher their evolutionary relatedness. These tools are discussed below:

16S rRNA sequence analysis

This is a culture free method of identifying and comparing microbial diversity from complex environment such as microbiomes that are difficult to study. It is amplicon sequencing method commonly used to identify bacteria or fungi present within a given sample down to the species level. 16S rRNA gene is approximately 1500bp long with 9 variable regions interspersed between conserved regions. These variable regions are used for phylogenetic classification of microbial population to genus or specie level. Sequencing is well established technique for comparing sample phylogeny and taxonomy. Sequence analysis of 16S ribosomal RNA (rRNA) specifies accurate typing of unknown isolates. History wise, this tool used for classifying organisms and evaluating their evolutionary relatedness was developed by Woese and coworkers in 1987.[55] This molecular approach has revolutionized the field of microbial biotechnology and has allowed meaningful phylogenetic relationships between microbes in natural environment to be discriminated.[56] Technically, polymerase chain reaction (PCR) is used to directly amplify the 16S rRNA gene

from sample colonies using universal primers which are directed at conserved regions at both ends of the gene. The PCR amplicon, about 1500 bp (1.5 kb) can then be directly sequenced and compared to the rRNA database for similarity checks.

The two major probiotic bacterial genera, *Bacteroides* and *Bifidobacterium*, are very heterogeneous and the use of 16S rRNA sequence analysis has contributed to the understanding of their phylogeny.[57,58] This is particularly important, as members of these genera are prime probiotic candidates for inclusion in probiotic cultures for both aquaculture and human consumption.

ITS sequence analysis

Internal transcribed spacer *ITS,* is the spacer DNA situated between the small subunit ribosomal RNA *rRNA* and large subunit *rRNA* gene in the chromosomes. In bacteria and archaea or fungi there is a single *ITS* located between structural ribosomal RNAs i.e. 16S and 23S rRNA genes. However, there are 2 types of *ITS* in the eukaryotes: - *ITS1* located between 18S and 5.8S rRNA genes, while *ITS2* is located between 5.8S rRNA and 28S rRNA genes. *ITS*1 corresponds to the *ITS* in bacteria and archaea or fungi. It's known that *ITS* region is highly used in sequence comparison, in taxonomy and molecular phylogeny. For instance, *ITS* markers have proven useful for clarifying phylogenetic relationship among different taxonomic groups.

ITS sequence analysis is a culture-free technique used in identifying bacteria or fungi which may not be possible using other methods. *ITS* sequence is a common amplicon sequencing methods for identifying and comparing microbes present within a given sample. Within the probiotic bacteria genus *Bifidobacterium*, the rRNA sequence is highly conserved[57] and may not be sensitive enough for the desired level of comparative analysis that is to be needed for selection of meaningful strains. Therefore, to complement the *rRNA* sequence approach, analysis of another molecule, which is not as conserved as *16S rRNA* but having the characteristics of a tangible phylogenetic marker is required.

The marker existing on the region between the 16S rRNA and 23S rRNA genes is termed as the internal transcribed spacer *ITS*. It has been used for more in depth analysis of probiotic strains especially *Bifidobacteria*.[57] In addition the *ITS* regions within the same bacterial strain can exhibit heterogeneity.[59,60] However, the molecule is precisely *PCR* compliance, as *PCR* can be used to amplify the molecule directly from sample colonies using universal primers directed at conserved regions within the adjoining area between *16S* and *23S rRNA* genes. Many authors have demonstrated the usefulness of *ITS* gene to assess phylogenetic relationship between cultivated species and their wild relatives. In some species, variation of *ITS* sequence has proven useful for studies at the population and species level due to its high sequence variations. Leblond-Bourget et al.,[57] evaluated the sequence analysis of this molecule for further characterising of *Bifidobacteria* and reported a great deal of sensitivity than the sequence analysis of rRNA. Furthermore, Tannock et al.,[61] also demonstrated its usefulness for identification of intestinal *Lactobacillus* spp.

recA gene sequence analysis

RecA is bacterial protein that functions to repair DNA damage and catalysis recombinant DNA repairs. Secondly it has a co-protease activity that regulates gene expression. The *RecA* system allows the identification of mutants with distinct split phenotypes. Recently, a short segment of the *recA* gene has been found to serve as a potential sensitive molecule for determining inter and intrageneric phylogenetic relationships. It is said to be found universally in bacteria and are also highly conserved. Furthermore, they are utilized in large scale analysis of a natural microbiomes or ecosystem, such as the GIT. This *recA* gene encodes the *RecA* protein, which plays vital roles in recombination as earlier stated.[62] Studies have shown that meaningful bacterial phylogenetic relationships can be determined by the sequence analysis of *RecA* protein.[63]

Eisen,[64] reported about the possibility of *RecA* gene segment serving as a useful molecule for phylogenetic analysis within a particular genus. This method was applied to the genus *Bifidobacterium* in a study by Kullen et al.[65] The gene was obtained from the *GIT* of *Bifidobacterial* isolates directly using *PCR* with primers directed to regions within the *recA* gene, yielding ~ 300 kb fragment which was sequenced using a single sequence reaction from either end. The sequence analysis showed phylogenic relationships of this short segment of *recA* gene, comparable with the analysis from the complete *rRNA* gene. Thus, given the rapidity with which the sequence information of this *recA* molecule was obtained, it is evident that *recA* gene sequence analysis could be potentially very valuable tool for comparative phylogenetic analysis of putative probiotic bacteria isolates.

Phenotypic fingerprint analysis

Fingerprint analysis has since been used to discriminate microbes at both phenotypic and genotypic level of inheritance. Although phenotypic fingerprints can be easily obtained, they are usually less sensitive and phenotypic changes may not necessarily translates to a different micro-organism, but rather could be due to or a reflection of changes in expression of the particular phenotype. Examples of phenotypic fingerprints analyses are serotyping, polyacrylamide gel electrophoresis of soluble gene analysis, fatty acid analysis, bacteriophage analysis etc. The most useful and rapid method of this procedure is serotyping. Here, colonies can be directly assessed by colony hybridization with a monoclonal antibody specific for a particular genus, strain or species without sub-culturing them. This approach was adopted by Corthier et al.,[66] and applied for the analysis of two *Bacteroides* species from different microbiomes.

Genotypic fingerprint analysis

The introduction of genotypic fingerprinting analysis for identification of diverse microbes from complex environments has been a major advantage for deciphering the complex microbes from this microbiome such as the GIT environment or ecosystem. The advent of this technique has contributed to our understanding of the diversity of microbes present in the GIT of different micro-organisms. Example of this molecular detection technique application was in a nucleic acid hybridization probe targeted at a specific DNA sequence. In addition to this rudimentary fingerprinting technique, more refined methodologies are currently in the making such as *Pulse Field Gel Electrophoresis.*

Pulse field gel electrophoresis PFGE

PFGE is a powerful genotyping technique essentially used for the separation of large DNA molecule with an electrical pulse system to migrate very large DNA fragments after digestion with unique restriction enzymes and on application to agarose gel matrix.[67] This technology can be applied to bacterial isolates after digesting its genome into relatively few segments. They can then be separated by *PFGE.*

With *PFGE,* the resulting segment of *DNA* fragments is always referred to as a restriction fragment length polymorphism *RFLP* and is highly sensitive and present exact attribute of the particular organism. The technique has been used by McCartney *et al*.,[68] and Kimura *et*

al.,[69] to examine the prevalence of *Lactobacilli* and *Bifidobacteria* in fecal samples over a period of time. They reported that this genomic technique offered one of the most discriminatory techniques in the history of molecular analyses.

RFLP of the 16S rRNA gene

This acronym *RFLP* refer to restriction fragment length polymorphism, is variations existing among microbial organisms in their *DNA* sequences at a site recognized by restriction enzymes resulting in differences in length of DNA fragments (this is produced by digesting the DNA with restriction enzymes).This involves rapid amplification technique for the *16S rRNA* gene using the *PCR* with primers targeted at the universally conserved regions within the gene. The resulting amplicon or *DNA* fragment is then treated with an appropriate restriction enzyme and the resulting restriction fragments are separated according to size by agarose gel electrophoresis, thus forming a characteristic *RFLP*.

*RFLP*s can be used as genetic markers, and applied in the analysis of traits inheritance in families. The choice of restriction enzyme depends on the particular genus of the organism and must be scientifically determined. *RFLP* is a *PCR* based technique and can be carried out as culture free method, thus eliminating the need of growing the isolates in culture medium. The discriminatory power of this technique focuses on the conserved nature of the gene concern. However, it gives the highest reproducible results.

Multiplex-polymerase chain reaction PCR

Multiplex-Polymerase Chain reaction *PCR* refers to the use of *PCR* to amplify several different DNA fragments concurrently using multiple primers. It involves the use of multiple primer sets within a single *PCR* sample mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By using this technique, additional information can be obtained from a single test that would otherwise require several reagents and more times to perform.

PCR can be used to amplify a specific DNA sequence from a single copy to over a million-fold using a thermostable DNA polymerase usually *Taq* DNA polymerase, deoxynucleotides (dNTP) and two primers, whose sequence is complementary to either end of the targeted *DNA* sequence. This could be accomplished by using multiple cycles of the PCR, generally 30 - 40. This molecular tool has been used successfully for the identification of *Lactobacillus* species.[70] Furthermire, it is also use in studying the diversity of organisms in the complex environment or intestinal microbiomes. With this, it will have a major impact on the field of probiotics development, as it application in deciphering the microflora of GIT in different organism is vital to understanding their functional role in intestinal health as probiotic candidates

Conclusions and suggestions for further work

Many microorganisms have been evaluated and selected as probiotics for use in aquaculture to include Gram-positive and negative bacteria, yeasts, fungi, microalgae and bacteriophages. However, there are some key issues which need to be investigated before any microorganism could be considered for use. This bordered on virulence and its ability to survive the heat of GIT and efficacy of the organisms. The contribution of this study to the field of probiotics is enormous, as rigorous scientific studies are the key to providing the necessary proof of potential probiotic organisms. The methodologies and techniques reviewed here are used potentially to explore the functionality of microbial traits in the putative probiotic organisms, particularly as molecular based techniques are becoming more sophisticated.

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Conflict of interest

Authors declare that there is no conflict of interest.

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