Next Generation Probiotics: Future Therapeutics for Sustainable Aquaculture

Shyam K.U.[1], Rahul Krishnan[2], Jeena K.[3], Vijaysunderdeva G.[1], Kurcheti Pani Prasad[4]

1 Ph.D. Scholars, 2 Research Fellow, 3 Scientist, 4 Principal Scientist, Aquatic Environment and Health Management Division, ICAR-Central Institute of Fisheries Education, Mumbai- 400 061

The live micro-organisms that are included in human diet or applied to the aquaculture systems are designated as traditional probiotics. According to FAO, traditional probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host. These live beneficial microorganisms are made use of as functional foods or food supplements. The journey of probiotics started since 1850 when Sir Louis Pasteur discovered lactic acid producing bacteria, the major spoilage organisms of milk followed by its isolation by Dr. Lister. Probiotic research is therefore 168 years old and has led to many findings and applications.

One smarter idea to find an alternative way to overcome side effects of chemical drugs is the use of engineered microbes or designer live microorganisms to produce or deliver therapeutics. This idea became reality through the discovery of next generation probiotics (NGP) or otherwise called live biotherapeutics (LBP) that have been designed to be used as living medicines to treat, cure or diagnose diseases, particularly in humans, that would be impossible with conventional probiotics.

Next generation probiotics

Currently used probiotic bacteria are drawn from a narrow range of organisms such as *Lactobacillus* spp., *Bifidobacterium* spp., etc. Advances in biotechnological research has lifted traditionally used probiotic bacteria to the next level or "next generation probiotics" (NGP). This primarily refers to those microorganisms which do not have the history of use as probiotics. An enormous amount of research on the microbial consortia (microbiome) of the gut and other body parts has enabled us to find new strains of microbes that do possess some probiotic functions. While cumbersome, this approach has resulted in paradigm shift to next generation level and as a result the probiotic era has moved on from using species-specific probiotics to strain-specific ones. NGP utilise the health promoting capabilities of specific bacterial strains.

NGP differ from traditional probiotics in that they are likely to be delivered under a drug regulatory framework. At present, conventional probiotics are used as either food ingredients or as supplements whereas NGPs are mainly used to treat or cure disease conditions in the body. Therefore, NGPs are considered as therapeutics (drugs) rather than functional foods or supplements, and hence must be delivered under regulations used for registering pharmaceuticals.

The US Food and Drug Administration (USFDA), defines live biotherapeutics (LBP) as biological products that contains live organisms, such as bacteria, and that are applicable to the prevention, treatment or cure of human diseases and are not vaccines. NGPs are generally considered to be LBPs except there are some operational differences, such as the former are usually identified and characterised by ongoing probiotic research laboratories based upon their microbiome research data, whereas the latter are developed as pharmaceuticals by business-oriented biotechnology start-up companies. Since NGP fall under the category of LBPs or drugs, any probiotic strain that will be used as NGP is subject to the usual pharmaceutical clinical trials and research on its pharmacokinetics, pharmacodynamics, safety and delivery routes. This process is stringent and poses significant hurdles on the pathway of NGP development and commercialisation.

To improve, the efficiency of organisms to be used as NGPs, certain scientific interventions, say to exclude toxic protein production or to improve their probiotic functions, are being made. This brings them into the category of genetically modified organisms (GMO). In the present scenario, the NGP industry is dominated by genetically modified bacteria designed to perform certain desired functions. As GMOs are a controversial topic these interventions in the probiotic sector may result in a reduced consumer preference. The scientific community is recommending that all NGPs should be included under the title of LBPs (drugs) so that consumer acceptance of conventional probiotics can be sustained.

Current candidate microbes for next generation probiotics

NGPs are more strain-specific than species-specific and it is important to know their health promoting features as probiotics. These features are related to their evolutionary history as in the case of traditional probiotics. For example, *Bacteroides fragilis* produces a toxic protein named fragilysin which is known to be a risk factor for human colorectal cancer. But a particular strain of *B. fragilis* strain ZY-312 is known to have immunomodulatory effect especially the phagocyte promoting activity, thus it can be considered a NGP.

Certain bacteria that doesn't have any probiotic function can also be utilised as live delivery vehicles for bioactive molecules or drugs. These are termed alternative NGPs. For example, *Lactococcus lactis* is normally not a probiotic bacteria but they are commensal to human gut. Researchers proved that they can be engineered to deliver some molecules that modulate inflammation such serine protease inhibitor, Interleukin-10 etc.

Some of the currently considered candidate strains for next generation probiotics are as follows:

Table 1. Characterised NGP/LBP candidates with their corresponding beneficial effect.

NGP Candidate	Effect on body
Bacteroides dorei D8	Convert cholesterol to coprostanol in vitro
Bacteroides acidifaciens	Increase IgA
Bacteroides ovatus	Increased levels of anti-TFα IgM and IgG antibodies.
Faecalibacterium prausnitzii	Induction of anti-inflammatory cytokines or reduction of pro-inflammatory cytokines

Development of NGP/LBPs

It's not easy to identify and introduce an NGP/LBP commercially. The development pathway is rigid and completely covered under the regulatory framework. According to USFDA regulations, a LBP should describe its complete characteristic features before it is applied. They are described as follows:

- Should describe the drug substance including the biological name and microbial strain details. The original source of microbe from which it derived inclusive of its culture history.
- Description about health status of clinical donor and a report of the phenotypic and genotypic expression of the bacterial strain. If we have done any genomic modification to the natural bacteria, a summary must be provided.
- Complete microbiological, biochemical and diagnostic characterisation of the strain including details about the antibiotic resistance.
- A manufacturer must be provided with complete and comprehensive data on mass scaling up manufacturing protocol (good manufacturing practices), infrastructure required and details of any other products produced in the same facility.

The major production challenges faced by the manufacturing sector includes the maintenance of anaerobic conditions in the whole production chain in order to maintain the viability and functionality of bacterial strains during the storage period. Since all identified NGP candidates are completely unable to grow in the presence of atmospheric oxygen (strict anaerobes) compared to microaerophilic traditional probiotics such as *Lactobcillus* spp. and *Bifidobacterium* spp., it is necessary to exclude the oxygen from the whole production line which is more technically difficult.

Pathway to produce a NGP/LBP

The commercial pathway to produce NGP/LBP and to obtain approval and it's commercialisation from the authority include identification of the NGP/LBP candidate bacterial strain, its complete characterisation, production and scaling-up of LBP and finally the clinical phase trials. Among all these steps, the most crucial and challenging task is the identification of suitable LBP candidates. Most of the time, researchers identify suitable strains based on some hypothetical approaches such as selecting an organism whose abundance level was depleted during alteration of the microbiome (dysbiosis of microbiome) in connection with any disease condition or on the basis of some organisms having an influence on a particular host pathway or phenotype relevant to a particular disease etc. An alternative is to screen blank unknown strains for a desired in vitro or in vivo probiotic activity.

Once a bacterial strain has been identified, the next stage is to characterise the LBP. This phase may include culture-dependent and culture-independent evaluations such as genome sequencing and screening for antibiotic resistance genes, toxic and virulent genes, enzymatic assays and so on. This step is also highlighted with the safety information regarding the strain, for that cell, animal and ex vivo model trials have to be conducted and documented.

The production phase encompasses the pilot and mass scaling-up of manufacturing protocols, establishment of defined media for the microbial culture, good manufacturing practices and formulation of effective delivery of the LBP stating its survival inside the host and bioavailability. The product approval will be commencing at the end of this phase and continues to the next phase. The usual pharmaceutical trials have to be conducted for commercial licensing and application. This includes three sub-phases: Phase 1 is a first in-human trial for confirming safety and dosage ranges and phase 2 and 3 are conducted on medium and large human populations respectively to know the efficacy, side effects and expanded safety in humans.

Unfortunately, the major challenges and issues faced by the last stage of the pathway is the approval and marketing. The authorisation of any micro-organism as a drug needs to be approved by the registered medical agency or council such as European Medical Agency, European Food Safety Authority (EFSA) in Europe and United States Food and Drug Administration (USFDA) in the United States.

Scope and applications of NGP/LBP

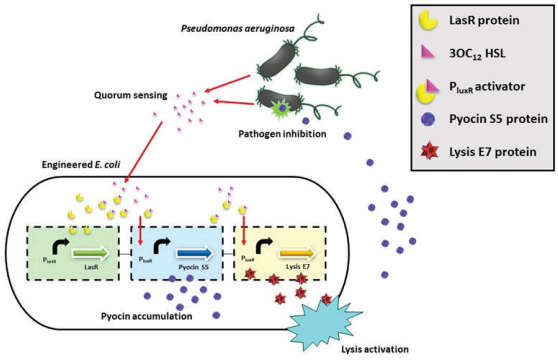
NGP/LBP has a plethora of identified and proven applications which signifies the new genera of probiotics. At present, in every field of science, the suitability of NGP/LBPs has been presented and continues to be evaluated. Some of the recognised fields include drug administration, vaccine delivery, immunomodulation (boosting the immune system), psychobiotics (for the treatment of mental disorders), cancer treatment and prevention, stress tolerance, production of antimicrobial compounds, to understand the pathogenesis of enteric infections.

The scope of NGP/LBPs lies in how we are designing probiotics by genetically engineering bacteria. Researchers are now applying synthetic biological tools and techniques to engineer NGP strains to address specific problems in human medicine and pharmacology. Some of those achievements are illustrated below.

1. Engineered therapeutics

Research findings were published in 2011 by Dr. Saeidi and his team from Nanyang Technological University, Singapore. They engineered probiotic *E. coli* (three additional genes were incorporated to the natural genome) to put the quorum sensing ability from *Pesudomonas aeruginosa*, a pathogen causing urinary tract infections and pneumonia in humans and connected it into the pyocin (a toxic protein produced by the *P. aeruginosa*) production. The mechanism they employed is that during infection, the *P. aeruginosa* will form a biofilm over the urinary tract (forms the quorum) and produces quorum sensing molecules to communicate between the bacteria. These molecules can be detected by the engineered *E. coli* (by production of LasR protein). Upon detection, *E. coli* will initiate the production of pyocin protein which accumulates inside the cell. With the help of another lysis protein, the cell wall is lysed allowing release of pyocin molecules. This inhibits the biofilm formation of *P. aeruginosa* (pyocin in high concentration is toxic to *P. aeruginosa* itself) (Illustration1).

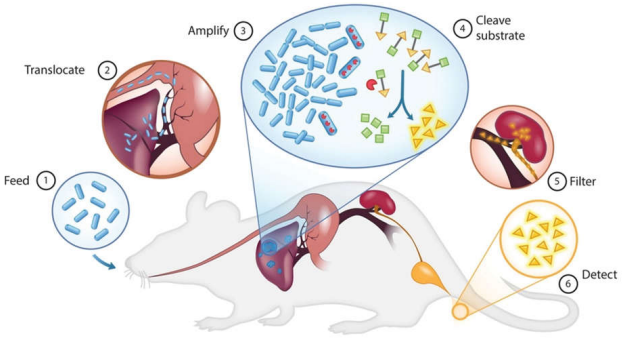
Illustration 1. After Saeidi et al., 2011



2. Living medical test

Apart from the duty of prevention and cure, another job that can be assigned to these designer probiotics is to detect specific diseases in body. An engineered probiotic can sense some disease-specific molecules from our body and give us the indication about the disease progression. One such living medical test was demonstrated by Danino et al. in 2015 to diagnose the early progression of liver cancer. They genetically modified a probiotic *E. coli* Nissle (ECN) strain by incorporating LacZ gene into the genome. The LacZ protein has the capacity to cut its respective substrate molecule to two parts. When engineered ECN was fed to mice (pre-clinical model), ECN was localised on progressive tumor tissues. LucGal (luciferin-galactose) substrate was injected into mice through a tail vein. Bacteria-produced LacZ protein specifically cut the LucGal substrate to luciferin and galactose. The by-product luciferin is filtered out through the urine which can be detected using a commercial luciferin-luciferase luminescent detection kit. Even 1 microliter of urine can be used to diagnose liver cancer via this living medical test (Illustration 2).

Illustration 2. From Danino et al., 2015



Scope of NGP/LBPs in aquaculture

Sustainability is a word we constantly use to describe aquaculture goals. Aquaculture farms often provide suitable conditions for diseases to flourish easily. The use and abuse of chemicals and antibiotics has been a problem in the aquaculture industry, raising concerns about food safety and development of anti-microbial resistant bacterial strains. Probiotics have proved their beneficial effects in disease management, stress control as well as in growth. If traditional probiotics are used as food supplements and as preventive measures for improving health, NGP/LBPs have the scope to treat or cure disease if introduced as an alternative to chemical drugs or antibiotics. Development of effective NGP/LBPs will be a promising potential step to sustainable health management in aquaculture.

Applications such as immunomodulation, stress tolerance, drug and vaccine delivery are fields where we can utilise the potential of NGP/LBPs in aquaculture. Increasing fish microbiome profiling and associated research can help to identify and characterise novel fish specific NGP strains. Interestingly, researchers have started to work on NGPs in the aquaculture sector by improving existing probiotic strains. The application of NGP/LBPs in aquaculture has not yet proceeded far. This may be due to the fact that LBP research is still in its infancy and may still be considered a bit controversial.

Conclusion

Next generation probiotics (NGP) or live biotherapeutics have opened up a wide array of possibilities of using live microbes as therapeutics and we can refer to them as therapeutic probiotics. They are a promising and eco-friendly alternative to high-impact chemical drugs and antibiotics, highlighting the fact that our own commensal bacteria can act as life-saving medicines to combat deadly diseases. There are some concerns regarding the NGP/LBP as they can involve genetically modified organisms. So future research should be directed towards the problems such as biocontainment of engineered probiotics and interactions between synthetic bacteria and commensal organisms in the gut. Data should also be generated on the aspects of interactions with the host's metabolic and signaling pathways and interactions with the aquatic environment in the case of aquaculture. We need to consider more than just the direct effects of these microbes on the system or host. Of course, we are still at a stage where more questions arise than answers regarding the next generation probiotics.

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