Review
A Review of Potential Implications of Probiotics for Human Health and \textit{in vitro} Selection of Probiotic Properties of Isolated Strains of Bacteria

Pranay Jain *, Vibhor Aggarwal

Department of Biotechnology, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra, India

* Author to whom correspondence should be addressed; E-Mail: drpranayjain@gmail.com.

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Abstract: Probiotic organisms are live microorganisms thought to be beneficial to the host organism. Lactic acid bacteria (LAB) and bifidobacteria are the most common types of microbes used as probiotics, but certain yeasts and bacilli may also be used. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures, such as in yogurt, soy yogurt, or as dietary supplements. Live probiotic cultures are available in fermented dairy products and probiotic fortified foods. However, tablets, capsules, powders and sachets containing the bacteria in freeze dried form are also available. They claim to help with health problems, ranging from constipation to diarrhea, and prevent colds or fight them. Probiotics are showing up in foods, beverages, and supplements. The application of probiotics as supplements in poultry has gained considerable interest during the last few years because antibiotic growth promoters (AGPs), added to animal feed to increase growth and decrease the incidence of diseases, are leaving harmful residues in meat and eggs. A wide range of microorganisms have been used as probiotics. For \textit{in vitro} selection of probiotic properties the strains are tested for their \textit{in vitro} antibiotics susceptibility, tolerance to bile, resistance to low pH values, acidifying activity, proteolytic activity, haemolytic activity, lactic acid and exopolysaccharide production, tolerance to stimulated gastric transit and bacterial viability during storage.

Keywords: lactic acid bacteria; probiotics; yogurt; intestinal contents; dietary supplements.
1. Introduction

Probiotics have been defined as “live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance” (Fuller, 1989). Among these microorganisms, lactic acid bacteria are regarded as a major group of probiotic bacteria (Collins et al., 1998). They are non-pathogenic, technologically suitable for industrial processes, acid fast, bile tolerant, adhere to the gut epithelial tissue and produce antimicrobial substances, including organic acids, hydrogen peroxide and bacteriocins (biologically active proteins) (Dunne et al., 1999). Growing human population urges the immense need to exploit the existing livestock resources to meet the animal protein requirements (Bilal, 2009). The concept of useful microbes is hundred years old when the people were in habit of consuming fermented milk. Lilly and Stillwell (1965), for the first time, used the word “probiotic” for this kind of microbes and described that probiotics are substances secreted by one microorganism that stimulate the growth of others. Fuller (1989) defined it as a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. Experiments into the potential health effects of supplemental probiotics include the molecular biology and genomics of Lactobacillus in immune function, cancer, and antibiotic-associated diarrhea, travellers' diarrhea, pediatric diarrhea, inflammatory bowel disease and irritable bowel syndrome (Ljungh, 2009).

2. Administration of Probiotics

The requirements for a microbe to be considered a probiotic are that the microbe must be alive when administered, it must be documented to have a health benefit, and it must be administered at levels to confer a health benefit. These are live microorganisms that will not provide the promised benefits if they don't stay alive. The manufacturer and consumer must pay close attention to the conditions of storage at which the particular microorganism will survive and the end of their shelf life. The other thing to remember is that these microorganisms are not all created equally. In fact, the genus, strain, and species all need to be the same for the results that you find in the study to be the results that you hope to achieve when taking it. For example, with the strain Lactobacillus rhamnosus GG, the genus is Lactobacillus, the species is rhamnosus and the strain is GG. If any one of those is different in your supplement, you may not attain the same results.

3. Health Benefits

Probiotics may seem new to the food and supplement industry, but they are available with human’s first breath. During a delivery through the birth canal, a newborn picks up bacteria from his/her mother. These good bacteria are not transmitted when a cesarean section is performed and have been shown to be the reason why some infants born by cesarean section have allergies, less than
optimal immune systems, and lower levels of gut microflora. Probiotics are believed to protect us in two ways. The first is the role that they play in our digestive tract. Our digestive tract needs a healthy balance between the good and bad bacteria. Poor food choices, emotional stress, lack of sleep, antibiotic overuse, other drugs, and environmental influences can all shift the balance in favor of the bad bacteria. When the digestive tract is healthy, it filters out and eliminates things that can damage it, such as harmful bacteria, toxins, chemicals, and other waste products. On the flip side, it takes in the things that our body needs (nutrients from food and water) and absorbs and helps deliver them to the cells where they are needed. The idea is not to kill off all of the bad bacteria. Our body does have a need for the bad ones and the good ones. The problem is when the balance is shifted to have more bad than good. An imbalance has been associated with diarrhea, urinary tract infections, muscle pain, and fatigue. The other way that probiotics help is the impact that they have on our immune system. Some believe that this role is the most important. Our immune system is our protection against germs. When it doesn't function properly, we can suffer from allergic reactions, autoimmune disorders (for example, ulcerative colitis, Crohn's disease, and rheumatoid arthritis), and infections (for example, infectious diarrhea, Helicobacter pylori, skin infections, and vaginal infections). By maintaining the correct balance from birth, the hope would be to prevent these ailments. Our immune system can benefit anytime that balanced is restored, so it's never too late (Modesto et al., 2009; Patterson and Burkholder, 2003; Tannock, 1995).

Experiments into the potential health effects of supplemental probiotics include the molecular biology and genomics of Lactobacillus in immune function, cancer, and antibiotic-associated diarrhea, travellers’ diarrhea, pediatric diarrhea, inflammatory bowel disease and irritable bowel syndrome ((FAO/WHO, 2002; Ljungh et al., 2009).

3.1. Diarrhea

Some probiotics have been shown in preliminary research to possibly treat various forms of gastroenteritis (King et al., 2003). They might reduce both the duration of illness and the frequency of stools (Allen et al., 2010). Probiotic treatment might reduce the incidence and severity of antibiotic associated diarrhea (Cremonini et al., 2002; D’Souza et al., 2002; Mcfarland et al., 2006; Sazawal et al., 2006; Szajewska et al., 2005 & 2006).

3.2. Colon Cancer

In laboratory investigations, some strains of LAB (Lactobacillus delbrueckii subsp. bulgaricus) have demonstrated anti-mutagenic effects thought to be due to their ability to bind with heterocyclic amines, which are carcinogenic substances formed in cooked meat (Wollowski et al., 2001). Animal
studies have demonstrated that some LAB have evidence for acting against colon cancer in rodents, though human data are inconclusive (Brady et al., 2000). Some human trials hypothesize that the strains tested may exert anti-carcinogenic effects by decreasing the activity of an enzyme called β-glucuronidase (Brady et al., 2000) (which can generate carcinogens in the digestive system). Lower rates of colon cancer among higher consumers of fermented dairy products have been observed in one population study, but confirmation of such an effect does not exist (Sanders et al., 2000).

3.3. Lactose Intolerance

As lactic acid bacteria actively convert lactose into lactic acid, ingestion of certain active strains may help lactose intolerant individuals tolerate more lactose than they would otherwise have tolerated (Sanders et al., 2000).

3.4. Blood Pressure

Although not a confirmed effect, some studies have indicated that consumption of milk fermented with various strains of LAB may result in modest reductions in blood pressure, an effect possibly related to the ACE inhibitor-like peptides produced during fermentation (Sanders et al., 2000).

3.5. Immune Function and Infections

Some strains of LAB may affect pathogens by means of competitive inhibition (i.e., by competing for growth) and there is evidence to suggest that they may improve immune function by increasing the number of IgA-producing plasma cells, increasing or improving phagocytosis as well as increasing the proportion of T lymphocytes and natural killer cells (Ouwehand et al., 2002; Reid et al., 2003). Clinical trials have demonstrated that probiotics may decrease the incidence of respiratory tract infections (Hatakka et al., 2001) and dental caries in children (Nase et al., 2001). LAB products might aid in the treatment of acute diarrhea, and possibly affect rotavirus infections in children and travelers' diarrhea in adults (Ouwehand et al., 2002; Reid et al., 2003), but no products are approved for such indications.

3.6. Cholesterol

Animal studies have demonstrated the efficacy of some strains of LAB to be able to lower serum cholesterol levels, presumably by breaking down bile in the gut, thus inhibiting its reabsorption (which enters the blood as cholesterol) (Sanders et al., 2000). A meta-analysis that included five double blind trials examining the short term (2 - 8 weeks) effects of a yogurt with probiotic strains on
serum cholesterol levels found a minor change of 8.5 mg/dL (0.22 mmol/L) (~4% decrease) in total cholesterol concentration, and a decrease of 7.7 mg/dL (0.2 mmol/L) (~5% decrease) in serum LDL concentration (Agerholm-Larsen et al., 2002).

3.7. *Helicobacter Pylori*

Some strains of LAB may affect *Helicobacter pylori* infections (which may cause peptic ulcers) in adults when used in combination with standard medical treatments, but there is no standard in medical practice or regulatory approval for such treatment (Hamilton-Miller et al., 2003).

3.8. Inflammation

Some strains of LAB may modulate inflammatory and hypersensitivity responses, an observation thought to be at least in part due to the regulation of cytokine function (Reid et al., 2003). Clinical studies suggest that they can prevent reoccurrences of inflammatory bowel disease in adults (Reid et al., 2003) as well as improve milk allergies (Kirjavainen et al., 2003). They are not effective for treating eczema, a persistent skin inflammation (Boyle et al., 2008). How probiotics may influence the immune system remains unclear, but a potential mechanism under research concerns the response of T lymphocytes to pro-inflammatory stimuli (Braat et al., 2004).

3.9. Side Effects

In some situations, such as where the person consuming probiotics is critically ill, probiotics could be harmful. In a therapeutic clinical trial conducted by the Dutch pancreatitis study group, the consumption of a mixture of six probiotic bacteria increased the death rate of patients with predicted severe acute pancreatitis (Basselink et al., 2008). In a clinical trial conducted at the University of Western Australia, aimed at showing the effectiveness of probiotics in reducing childhood allergies, researchers gave 178 children either a probiotic or a placebo for the first six months of their life. Those given the good bacteria were more likely to develop sensitivity to allergens (Bee et al., 2008). Probiotics taken orally can be destroyed by the acidic conditions of the stomach. A number of micro-encapsulation techniques are being developed to address this problem (Islam et al., 2010). Recent studies indicate that probiotic products such as yogurts could be a cause for obesity trends (Raoult et al., 2009). However, this is contested as the link to obesity, and other health related issues with yogurt may link to its dairy and calorie attributes (Ehrlich et al., 2009; Raoult et al., 2009).

4. *In vitro* Selection of Probiotic Properties

4.1. Antibiotic Resistance
Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. While a spontaneous or induced genetic mutation in bacteria may confer resistance to antimicrobial drugs, genes that confer resistance can be transferred between bacteria in a horizontal fashion by conjugation, transduction, or transformation. To do this, a pure strain of bacteria is isolated. This pure strain is then spread over the surface of a special medium, called Mueller-Hinton agar, to create a lawn, or carpet, of bacteria. Small filter paper discs, impregnated with standardized amounts of antibiotic, are gently pressed on to the surface of the agar. While the plates are incubating overnight, the antibiotic diffuses from the disc and into the agar. This antibiotic diffusing into the agar will inhibit the growth of susceptible bacteria. Then the zone of inhibition is measured using zone scale.

4.2. Bile Tolerance

Once the bacteria reach the intestinal tract, their ability to survive depends on their resistance to bile (Gilliland et al., 1984). Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria. Hence, the success of a probiotic also depends on the bile-resistant qualities of the selected strains. The method used for testing bile tolerance is described by Gilliland et al. (1984). The bacteria are grown overnight in MRS broth and then 2% freshly-prepared overnight cultures are inoculated into tubes containing MRS broth with 0.3% ox gall, MRS broth containing 7 mM sodium taurocholate, and MRS broth without bile (as controls). The inoculated tubes are incubated at 42 °C. Growth is monitored at 2h-interval for 24 h (OD600). Bacterial growth is expressed in colony forming units per milliliter (cfu/mL) and the survival percentage (% ± sd) of strains to bile is then calculated. All tests should be carried out in triplicate.

4.3. Acid Tolerance

The bacteriocin producing LAB isolates may grow in MRS broth at 30 °C without shaking and growth and have to be monitored at 650 nm. Adaptation to low pH: Dilute log-phase LAB cultures 100 fold in fresh MRS broth (10 mL) to make a preculture. Dilute again this preculture 100 fold in 100 mL fresh MRS broth. Grown this culture to cell density $5 \times 10^8$ cells/mL (OD650 = 0.5). Harvest bacteria by centrifugation (10,000 rpm, 5 min). Resuspend cells in an equal volume of MRS broth (devoid of yeast extract) having pH 5.5 and 6.0. Allow to adapt for 30 min at 30 °C.

Acid challenge: Harvest cells by centrifugation and resuspend in an equal volume of MRS broth adjusted at pH 3, 2.5, 2 and 1 using 1 N HCL. Determine counts of viable cells after 0, 15, 30, 60, 120 min and also after 12 and 24 h. Dilute samples in peptone water and determine viable counts by plating on MRS agar and incubation at 30 °C for 6 days.
4.4. Lactic Acid Production

The method that can be used on the bacteria is based on the detection of lactic acid produced from lactose utilisation. Bacteria are grown in a broth where the only carbohydrate source is lactose. Subsequent growth will be dependent upon the ability to ferment lactose and thus will be dependent upon the presence of β-galactosidase (lactase). Fermentation of lactose will result in the production of lactic acid. The presence of lactic acid will result in a drop in pH which will subsequently cause the phenol red indicator to change to yellow. Production of lactic acid can also be measured by precipitate method according to Pryce (1969) and expressed in mmol/L.

4.5. Acidifying Activity

Acidification can be measured by the change in pH (ΔpH) during time according to Lombardi et al. (2002) and Ayad et al. (2004) methods. Fifty milliliters of MRS is inoculated with 2% of culture and incubated at 37 °C. The pH is measured at 0, 2, 4 and 6 h using a pH-meter. The acidification values are then expressed as pH decrease, calculated as the difference between the value immediately after inoculation and values at 0, 2, 4 and 6 h (ΔpH = pH at time - pH zero time).

4.6. Proteolytic Activity

The proteolytic activity of strains can be determined in skimmed milk medium (reconstituted skimmed milk powder 10% w/v) by the tyrosine method (Hull, 1947), in accordance with International Dairy Federation (IDF) standard 149A (1997). Milk is inoculated at 0.2% with strain precultured in MRS broth at 37 °C for 18 h, to obtain approximate $10^6$ cfu/mL and then incubated at 30 °C for 24 h. The IDF method is based on the reaction of the amino acids tyrosine and tryptophan realised from the milk substrate at 72 h with a phenol reagent, yielding a blue colour that is measured at 650 nm. The results are calculated from a calibration curve obtained from dilutions of tyrosine in distilled water and expressed as mg tyrosine/liter of milk.

4.7. Exopolysaccharide Production

Exopolysaccharide production can be evaluated as reported by Mora et al. (2002). Overnight cultures were streaked on the surface of plates containing ruthenium red milk (10% w/v, skim milk powder; 1% w/v, sucrose; 0.08 g/L ruthenium red; and 1.5% w/v, agar). After incubation at 37 °C for 24 h, non-ropy strains will give red colonies due to the staining of the bacterial cell wall, while ropy strains will appear as white colonies.

4.8. Haemolytic Activity
For hemolytic activity tests, the strains are subcultured in MRS or TPY and then streaked on Columbia agar plates, containing 5% of sheep blood. The plates are incubated for 24 h at 37 °C in anaerobic jars. As suggested by Maragkoudakis et al. (2009), the strains that produced green-hued zones around the colonies or do not produce any effect on the blood plates are considered non-hemolytic. The strains showing blood lysis zones around the colonies are classified as hemolytic (β-hemolysis). Experiments are to be performed in triplicate.

4.9. Antibiotic Resistance Activity

Antibiotic resistance assays are performed with: tetracycline, trimethoprim, cefuroxime, kanamycin, chloramphenicol, vancomycin, ampicillin, streptomycin and erythromycin. Serial dilutions of antibiotics are prepared in water to reach concentrations in the range 1 - 512 μg/mL. The assay is performed in 96 well plates; in each well 20 μL of appropriate dilution of antibiotic, 160 μL of fresh MRS or TPY broth and 20 μL of overnight bacterial suspension previously diluted in fresh MRS or TPY broth to obtain 10^6 cfu/mL are added. Growth or inhibition of the strains is determined by measuring the A620 at regular time intervals for a total incubation of 24 h at 37 °C. The sensitivity of the strains to each antibiotic is expressed as the minimal inhibitory concentration (MIC). The choice of antibiotics and the characterization of sensitivity/resistance are according to the guidelines and breakpoints of the European Commission (EU commission, 2002) and EFSA (EFSA, 2005).

4.10. Tolerance to Stimulated Gastric Transit

Tolerance of isolated strain to stimulated gastric transit can be determined, as described by Dunne et al. (2001). For this purpose, isolated bacterial culture is mixed with 3 mL of stimulated gastric juice and 1 mL of phosphate buffer saline at the rate of 10^5 CFU. Bacterial survival is evaluated after 30, 60, 90 and 120 min of incubation.

4.11. Bacterial Viability during Storage

The storage viability of isolated species has to be recorded weekly at -20 °C, 4 °C and room temperature. The test tubes are to be inoculated with 10^5 CFU of culture suspension. These inoculated test tubes are to be stored at -20 °C (with 10% V/V glycerol), 4 °C and room temperature for 6 weeks. The growth has to be monitored weekly by plate count method.

5. Conclusions

The beneficial effects of probiotics on animal health and on the reduction of pathogens in the food chain have increasingly been highlighted in the past years. With *in vitro* studies according to FAO
and WHO guidelines probiotic potential of selected LAB and bifidobacteria strains can be evaluated to assess the capability of the most promising strains to colonize the GI tract of poultry, and human beings. The probiotics finally can be used as additives in feed for livestock poultry in order to reduce C. jejuni contamination and therefore enhance the safety of poultry meat. These probiotic fermented foods claim to help with health problems, ranging from constipation to diarrhea, and prevent cold or fight them. Probiotics are showing up in foods, beverages, and supplements. The application of probiotics as supplements in poultry has gained considerable interest during the last few years because antibiotic growth promoters (AGPs), added to animal feed to increase growth and decrease the incidence of diseases, are leaving harmful residues in meat and eggs.

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