Revista Latinoamericana de Microbiología (1998) 40:166-172
Asociación Latinoamericana de Microbiología

A Review on the Use of Microorganisms as Probiotics.

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ABSTRACT. A literature review on the use of live microorganisms as probiotics is presented. Topics discussed are the definition of probiotic; the normal microflora of the digestive system of mammals, including bacterial interactions in the gut, colonization, modification of metabolic processes, and immunostimulation. Probiotics studies in humans and in farmed animals, with special emphasis on the use of *Lactobacillus* spp. and *Bifidobacterium* spp. are also discussed.

RESUMEN. Se presenta una revisión bibliográfica sobre el uso de microorganismos vivos como probióticos. Se revisan temas como la definición de probiótico; la microflora normal del aparato digestivo de mamíferos, incluyendo interacciones bacterianas, colonización, modificación de los procesos metabólicos e inmunoestimulación. También se revisan los estudios sobre probióticos realizados en humanos, con especial énfasis en *Lactobacillus* spp. y *Bifidobacterium* spp.; así como en animales de granja.

DEFINITION

Elie Metchnikoff's work could be regarded as the first research conducted on probiotics at the begining of this century.[18] He described them as "microbes ingested with the aim of promoting good health". This same definition was modified to "organisms and substances which contribute to intestinal microbial balance",[47] and later by Fulle[18,19] "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". These definitions were originally developed for farm animals or humans, since the first studies on the subject were carried on these species. Probiotics are now also being used in aquaculture and therefore the definition may have to be changed to include a possible improvement of the environmental water quality.

Probiotics cannot be classified as a type of biological control, as the definition of biological control is the utilisation of natural enemies to reduce the damage caused by noxious organisms to tolerable levels[10] or more strictly, the control or regulation of pest populations by natural enemies.[60] A probiotic microorganism does not directly attack the noxious agent (pathogen) but merely prevents its action on the host; at most, it may produce substances that inhibit the harmful organism. Neither should probiotics be identified as growth promoters, since their action might not be associated with growth of the host but a general improvement in health, growth may be only one of the effects. Prebiotic is related term, which has been used to define a non-digestible food which improves the growth of bacteria in the colon.[71]

THE MICROFLORA OF THE DIGESTIVE SYSTEM

The human foetus inside the uterus is sterile, but as soon as it passes through the vagina during birth, it acquires vaginal and faecal microorganisms.[20] The gut flora is acquired from its immediate environment, establishes rapidly and is characteristic of its species.[30,19,61] The initial period of bacterial colonisation in the new-born human takes place normally in a two week period. During this time, breast-fed and bottle-fed infants harbour similar bacterial species in the colon. *Escherichia coli* and *Streptococcus* spp. are almost always the first species to appear, at concentrations between 10^8 and 10^{10} organisms/g of faeces.[21] After that time, gross differences exist depending on the infant's feeding. Breast-fed infants harbour mostly *Bifidobacterium* spp. while bottle-fed ones show a more varied flora consisting, among others, of *Lactobacillus* spp.[1], *Bacteroides* spp., *Clostridium* spp. and *Streptococcus* spp.[44,20] This difference is maintained until other foods are incorporated in the diet.

After weaning (>2 years), the final flora in humans is established and can become a very complex collection of approximately 10^{14} microorganisms consisting of more than 400 different types of bacteria.[42] The large intestine can contain up to 10^{12} bacteria/g of gut content.[20] The gut of other species has been found to contain similar numbers of bacteria, 10^{11} cell/g in the caeca of poultry,[2] 10^{10} bacteria/ml in the rumen of ruminants.[67]

The majority of the flora in the large intestine of humans is strictly anaerobic, where the predominant genera are *Bacteroides* and *Fusobacterium*; other genera also found are *Bifidobacterium* spp., *Clostridium* spp., *Lactobacillus* spp., several Gram-positive rods belonging to the eubacteria and other Gram-positive cocci.[1,20] The stomach and upper small intestine are populated by a low number of bacteria because of the acidity of the contents.[37,21]

BACTERIAL INTERACTIONS

Microbial interactions represent the main force that contributes to the homeostasis of the bacterial flora in the gut.[52] The components of the gut ecosystem involve a) biotic, microbes and gut lining; b) abiotic, feeds; and c) endogenous components, secretions of the organism and others. Gastrointestinal problems are often associated with destabilisation of the ecosystem.

The most likely form of interaction when two bacterial species or strains share the same environment, is antagonism.[17] Therefore, one kind of bacteria will reduce or eliminate the other strain. This concept is important in the probiotics theory, but unfortunately, deciding which species of bacteria is appropriate for use as a probiotic has been principally a matter of intuition rather than scientific approach.

COLONISATION

The gut microflora consists of microorganisms that permanently colonise the tract as well as others that are transient. If they are to survive and colonise the gut, they have to withstand the peristaltic movement, which tends to flush the gut. They can overcome this either by multiplying rapidly or by attaching themselves to the gut wall.[30,19,26] This may involve free association in the lumen or colonisation of epithelial surfaces, either through adherence to structures on the surface or colonisation of secretions overlying the epithelial cells.[56]

Adherence can be considered the first step of colonisation, but after settlement the organism must be able to multiply, in the face of host defence mechanisms and interactions with the surrounding microflora. Attachment of bacteria is very species dependent, and bacteria isolated from one animal may not be able to colonise similar sites in another animal species,[30,26] therefore, colonisation is a host specific phenomenon that can be influenced by the particular growth conditions.[19] Attachment to the squamous epithelium of the stomach, for example, shows a high degree of specificity.[16] Bacteria must be also capable to resist the action of the bile and other potentially harmful substances characteristic of the gut, the anaerobic conditions and be able to utilise the available nutrients.[61]

The microbial flora inside the gut was believed since long time ago to have adverse effects on the host organism (Metchnikoff's hypothesis), but soon researchers realised that it was also involved in the protection of the host and contributed to its nutrition.[18] A major concept in the probiotic theory is that the indigenous microflora of humans and animals provides protection against infections with pathogenic microorganisms.[16,19,27] One mode of action of probiotic bacteria is by protecting the host through colonisation of the gut, this phenomenon has been called "barrier effect", "bacterial antagonism", "bacterial interference", "colonisation resistance" and "competitive exclusion".[18,19,50] Barrier effects are extremely efficient for preventing intestinal infections and for maintaining some bacterial strains at a low population level. It has been proven that enterotoxin producing strains of *E. coli* can only induce diarrhoea through attachment to the intestinal wall.[59] Therefore if a beneficial strain colonises the gut lining it will prevent this enteric bacteria from causing disease, thus acting as a barrier against the colonisation of the pathogen.

The best evidence for the protective effect of the gut flora stems from the observation that animals free of microorganisms are more susceptible to disease than are the ones with a complete intestinal flora. Some of the crucial work that aided in the development of this idea was done by Bohnoff *et al.*[4] They showed that by administering antibiotics to experimental mice, they rendered them more susceptible to *Salmonella typhimurium* infection. Similar results have been obtained by other workers testing *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in mice.[66] Collins and Carter[6] showed that microorganism-free mice could be killed with 10 cells of *Salmonella enteriditis*, but 10^9 cells were required to kill an animal with a complete gut microflora.

MODIFICATION OF METABOLIC PROCESSES

Another way by which probiotic organisms may be beneficial is by modifying metabolic processes, as summarised by Rowland.[53] These modifications could be accomplished in several ways.

a) By suppressing reactions which result in the generation of toxic or carcinogenic metabolites. The degradation of nitrosamines by lactobacilli and *E. coli* strains[54] is an example.

b) By stimulating enzymatic reactions involved in detoxification of potentially toxic substances. An example is the reduction of amine production in the gastrointestinal tract.[50]

c) By stimulating mammalian enzymes involved in the digestion of complex nutrients. Probiotics might influence the activity of useful enzymes such as ß-galactosidase.[23]

d) by synthesising vitamins and other essential nutrients not provided in sufficient quantities in the diet.

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IMMUNOSTIMULATION

The stimulation of the immune system can be achieved by increasing the antibody levels and/or by augmenting the macrophage activity.[19,48] Evidence for this is that normal animals with a complete gut flora have increased phagocytic and immunoglobulin (IgA) levels compared with gnotobiotic animals.[19] Yoghurt, with its lactic-acid bacteria, is capable of inhibiting the growth of intestinal carcinoma through increased activity of IgA, T cells and macrophages[48] which have immunostimulatory and antimutagenic properties.[13] Also whole cell extracts of certain lactic acid-producing strains are able to alter macrophage function.[25]

The normal microbial flora of an animal has a significant impact on the body's immune system.[16] Specifically, they can improve intestinal immunity by adhering to intestinal mucosa and therefore stimulating local immune responses.[55]

PROBIOTIC STUDIES IN HUMANS

Metchnikoff employed a species of *Lactobacillus*, the so called "Bulgarian bacillus" isolated from yoghurt starter cultures, most probably *L. delbreuckii* subsp. *bulgaricus*. He tried to relate long life with the consumption of fermented milk, since he found that Bulgarian peasants who ingested large amounts of soured milks, lived to an old age.[18,8] He thought that the ingestion of bacteria had a positive influence on the normal flora of the gastrointestinal tract.[28] Fermented dairy products have been reported to be effective in the treatment of a number of disorders, including diarrhoea, colitis, flatulence, gastric acidity, gastroenteritis, constipation and neoplasia.[21,51] So, it is not surprising that microorganisms involved in the fermentation process have been selected as potential probionts.

Since Metchnikoff studies, several other species have been used; among them are up to 10 species of *Lactobacillus*, 6 of *Bifidobacterium*, *Streptococcus salivarius*, *Enterococcus faecium*, *Lactococcus lactis*, *Leuconostoc sp.*, *Pediococcus sp.*, and *Bacillus sp.* Yeasts have also been examined, such as *Saccharomyces cerevisiae* and *Candida pintolopesii*; and even fungi as *Aspergillus niger* and *A. oryzae*.[19,28,36] A review of the principal species employed is presented below.

Lactobacillus spp.

The species of bacteria used by Metchnikoff (*Lactobacillus delbreuckii*) had been employed for the preparation of yoghurt, but was found to be incapable of colonising the intestinal tract of humans and animals.[8] Other *Lactobacillus* species where also found to be incapable of such colonisation.[40] It is important to remember that adherence and colonisation are very important factors for an effective probiotic.[37,26] Another species used is *L. acidophilus*, of which some strains are capable of resisting the adverse conditions of the human gut[43] and passing through the entire digestive tract[38,57,39]and to colonise it.[29] *L. reuteri* was tested for its ability to colonise and it was demonstrated that it could establish after seven days of administration and could persist for another seven days after end of administration.[69] *L. plantarum* had also a good colonisation capacity, according to results by Bengmark and Jeppsson.[3] *L. gassei, L. casei L. fermentum* and *Bifidobacterium* have also been shown to adhere to the intestinal cells.[29,62]

L. acidophilus was originally employed since it was thought to be the dominant lactobacillus in the intestine, but later work showed that there is a wide range of species, such as *L. casei, L. reuteri, L. salivarius, L. fermentum, L. cellobiosus* and *L. plantarum*.[37,63] The justification for Lactobacilli being the most commonly used group is the evidence that just after birth, a decrease in numbers of certain bacteria was observed when *Lactobacillus* increased.[19] This genus and *Streptococcus* are the two genera that appear most frequently in effective probiotic preparations.[49,51]

A mixture of *Lactobacillus* spp. supplied with fermented milk shows a therapeutic effect in children affected by diarrhoea.[22] Lactobacilli also have been found to improve the interferon-alpha producing capacity of the immune system[33] and specifically the gut immune system.[8,11,55]

Another factor in favour of lactic acid bacteria, is their capacity to inhibit various pathogenic Gram-negative bacteria, due to the production of "bacteriocin-like substances".[5] *L. acidophilus* produces acidolin, acidophilin and lactacin B, all antimicrobials capable of inhibiting many Gram-positive and Gram-negative bacteria including *Staphylococcus* spp., *Salmonella* spp., *Shigella* spp. and *Pseudomonas* spp.[26] *Lactobacillus* also produces large amounts of lactate from simple carbohydrates and therefore can withstand a high degree of acidity which may usually inhibit other bacteria.[30] Later analysis showed that simple carbohydrates do not reach the lower part of the gut because they are absorbed in the upper parts. Nevertheless, *Lactobacillus* can modify intestinal bacterial metabolism producing beneficial effects.[53]

The use of *Lactobacillus* species may also have some negative effects. Studies have shown some potential pathogenic activity of *Lactobacillus* spp. (e.g. dental caries, rheumatic vascular disease and septicaemia). They have also recently been identified as potential emerging pathogens in elderly and immunocompromised patients, particularly those receiving broad-spectrum antibiotic therapy.[37,24] Elmer[15] found no evidence that administration of selected microorganisms is beneficial in the prevention and treatment of certain intestinal and, possibly, vaginal infections. In contrast, several authors have found the use of this bacteria as negligible or no treat to human health. Saxelin[58] proved that eight strains of *Lactobacillus* isolated during a four-year study of bacteremic patients had a very low pathogenic activity. Huis In't Veld[29] made a critical analysis of data supporting the potential pathogenicity of lactic-acid bacteria and concluded that there is a negligible risk in the consumption of this bacteria and recalled that they have a "generally recognised as safe" (GRAS) status.

Bifidobacterium spp.

As mentioned earlier, *Bifidobacterium* spp. were found to be the dominant flora in breast-fed infants and not common at all in formula-fed ones.[44] Also it was noted that breast-fed babies were more disease resistant. These findings led to the idea that this bacterial flora gave the infants some protection. Bifidobacteria are also able to withstand the conditions of human gut.[38] Therefore this bacteria are also used as a probiotics. The species used are *B. adolescentis, B. animalis, B. bifidum, B. longum, B. breve, B. infantis,* and *B. thermophilum.*[8,50,20]

Daly,[8] Marshall[37] and Gibson and Roberfoid[20] claim that the principal positive effects of *Bifidobacterium* species are a) reduced blood ammonia levels, b) lowered blood cholesterol levels, c) growth suppression of potential pathogens, d) restoration the normal intestinal flora following antibiotic therapy, e) synthesis of vitamins, f) immunomodulation, g) anticarcinogenic activity, h) improved lactose utilisation. There are reports of *Bifidobacterium dentium, B. inopinatum* and *B. denticolens* as being the cause of dental caries.[7]

PROBIOTIC STUDIES IN ANIMALS

A young animal acquires its protective microflora from its mother and/or from the environment;[19] for example the hen excrement is the major source of inoculant intestinal bacteria for the newly hatched chick, or the intimate contact of the piglet with the sow.[16] Therefore, under normal conditions there is no need for the administration of probiotics. The flora can be affected by change in diet, antibacterial drugs and stress. When there is a need to provide probionts to animals, they are administered in powders, pastes or tablets directly into the mouth or mixed with the feed. One dose has proved often to be insufficient, so several doses are advised.[18]

Probiotics have been used in farm animals as growth promoters, to replace antibiotics and synthetic chemical feed supplements, protection for intestinal infections, and to reduce susceptibility to stress.[61,19]

Poultry

Studies have been carried out with broilers, laying hens, turkeys, bobwhite quail, geese and ducks; but research has focused mostly on chickens.

The normal bacterial flora of the chicken is almost constant throughout the length of the gut and it is composed mainly of *E. coli, Clostridium* spp., *Lactobacillus* spp. and several other anaerobes.[2] *Salmonella* spp. infection is a serious disease in chickens and also a potential human health risk,[2] but the colonisation of the gut by *Salmonella* spp.,[70] *E. coli*[16] and other enteropathogens[2] can be prevented by competitive exclusion with the aid of probiotics.

As growth promoters, probiotic supplementation of chicken diets has been variable but some papers report a statistical significant effect on growth and egg production;[16,19,34] nitrogen retention and lower cholesterol[41] with *Lactobacillus* spp. Broilers also showed an increase in weight gain and better feed conversion when they were fed with *Lactobacillus acidophilus* in the drinking water.[64] Other authors, reviewed by Jernigan,[30] found no significant differences in the results when a probiotic was added to the diets of broilers or in the production of eggs, or at best, small improvements were obtained. Difficulties in demonstrating statistical significant differences have been due to microbiological variation in the gut flora of individuals and different groups of animals.[2]

Pigs

One of the mayor causes for mortalities among piglets is diarrhoea, accounting for up to 41% of the total deaths and most cases are caused by enterobacteria[16,32] such as *E. coli.*[62] Many studies on probiotics have focused on preventing the infection by these bacteria in neonatal or young pigs (weaning stage); it is during this period that the pigs are more susceptible.

Lactic-acid bacteria are the group most commonly employed as probiotic in pigs, where a large proportion of the gut microflora consists of *Lactobacillus* spp.[32] They have been tested in all growing stages of the pig. Several authors (compiled by Fox[16]) have proven up to 53% reduction in mortality when pigs were fed with *Lactobacillus* spp. compared to untreated animals. Reduction of enterobacteria (*E. coli*) and the diarrhoea associated with it has been achieved with the administration of *Lactobacillus* spp.[9,35,65]

Ruminants

Ruminants is perhaps one of the groups of farmed animals where less probiotics studies have been done. During the early life of the ruminants food tends to bypass the rumen with a gradual change to microbiologic fermentation in the rumen after weaning. Therefore there are two stages of bacterial colonisation and two very different microbial populations.[67] Most probiotic work has focused on young animals.

Lactobacillus acidophilus has been the probiotic of choice[31] and it has reduced the coliform numbers[14] and thus prevented diarrhoea.[16]

The yeast *Saccharomyces cerevisiae* has been used to stimulate the bacterial fermentation in the rumen because with its respiratory activity greatly reduces oxygen concentration, thus permitting anaerobic bacterial metabolism.[46] Yeast also promotes cellulolytic bacteria,[45] total anaerobic, amylolytic, pectinolytic, xylanolytic bacteria and enterobacteria.[31] In some of these experiments no statistical differences were demonstrated. Yeast, however, permitted an increased milk production in dairy cows by as much as 1.4 litres/day.[68]

CONCLUSIONS

Many species of microorganisms have been used as probiotics, but *Lactobacillus* and *Bifidobacterium*, specially the first, are by far, the genera most commonly employed in humans and farmed animals.

Probiotic organisms must have one or several modes of action. They can prevent potential pathogenic organisms to become established in the digestive tract, they can also modify metabolic processes within the digestive tract, stimulate the immune system and produce inhibitory substances. If a probiotic is to accomplish some of these tasks, it has to successfully colonise the internal environment.

The available data does indicate that living microorganisms can protect and enhance the performance of treated animals. The most dramatic results have been produced in animals, which have lost their normal intestinal flora due to antimicrobial treatments.

Some data also suggests a potential pathogenic effect of certain *Lactobacillus* species in elderly and immunocompromised patients, although lactic-acid bacteria have a "generally recognised as safe" (GRAS) status.

Unfortunately, much of the research conducted on probiotics in mammals is of very low scientific value, since most of the experiments lack replicates, controls or a good experimental design.

ACKNOWLEDGMENTS

This work was partially funded by the International Foundation for Science, project A/2203-1.7.

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