sciendo

DOI: 10.2478/fv-2019-0039

FOLIA VETERINARIA, 63, 4: 60—69, 2019

OPTIMAL CRITERIA FOR THE SELECTION OF PROBIOTICS, BASED ON THEIR MODE OF ACTION

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ABSTRACT

The objective of this review was to discuss some of the criteria which influence the selection of microorganisms with probiotic properties based on their mode of action. The most common bacteria that belong to the "group" probiotics are the *Lactobacillus* and *Bifidobacterium* species/strains. Probiotics have benefits and effects by their mechanism of action in different axial locations such as: producing substances, influencing immune function and response, modification as well as maintenance of a healthy population of microorganisms in the intestinal environment. Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases Potential peripheral pathways that link probiotic ingestion in the brain function are focused on the role of the vagal afferent nerve signalling and changes in the cerebral levels of neuromodulators. The application of probiotic microorganisms represents a way to effectively influence the composition of the intestinal microbiome and the immune system of the host, as well as they can be considered as a suitable alternative to influence a healthy quality of life.

Key words: additives; immunity; intestinal tract; probiotic; properties; selection; technology

INTRODUCTION

The administration of live organisms is not without risk, particularly in certain populations. The important question is to determine if the health benefits of probiotics or even components/products of these agents can be successfully attained without the risks associated with the administration of a live organism to a host [31].

The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria. Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases, but the mechanisms responsible for these effects have not been fully characterized yet. Several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune

system to convey an advantage to the host [5]. By adhering to the alimentary tract, probiotic organisms may survive difficult conditions, and offer a beneficial effect on the stability and protection of the intestinal environment. They also influence the course of digestive and metabolic processes and the immunological response, leading to an improved health and an increased productivity of the animals [28].

Lactobacillus, Bifidobacterium, Enterococcus and several other microbial species are perceived to exert such effects by changing the composition of the gut microbiota [32]. The mucus layer of the intestinal tract plays an important role in forming the front line of innate host defence. The involvement of natural substances feeding on protection/prevention/promotion of mucus production in the intestinal environment is beneficial. The intestinal mucus forms enterocytes covered by transmembrane mucins and goblet cells secreting by the secreted gel-forming mucins (MUC2). The goblet cells continually produce mucins for the retention of the mucus barrier under physiological conditions, but different factors (e. g. microorganisms, microbial substances, viruses, cytokines, enzymes etc.) can have profound effects on the integrity of the intestinal epithelium covered by a protective mucus gel composed predominantly of mucin glycoproteins [59].

The microbiota, the intestinal epithelium, and the mucosal immune system constitute the gastrointestinal ecosystem. All three components are essential for the complete function and development of the system. Probiotics can influence immune function through a number of different pathways including effects on enterocytes, antigen presenting cells including both circulating monocytes and local dendritic cells (DC), regulatory T cells, and effector T and B cells. The mechanisms of action of probiotics involve modification of the microbial population, aggregation with pathogenic bacteria, competitive adhesion to epithelial receptors, competition for nutrients, modification of the structure and function of the intestinal epithelium and production of specific substances (e. g. bacteriocins, organic acids—lactic acid, hydrogen peroxide, biosurfactants, adhesion inhibitors, co-aggregation molecules) [63]. Certain probiotic microorganisms can enhance the function of the intestinal barrier through modulation of the phosphorylation of cytoskeletal and tight junction proteins and thereby influencing the intestinal mucosal cell to cell interactions and cellular "stability" [48].

Probiotics—definition and the mode of action

The definition of "Probioticum" was formulated in 1974, simultaneously with the use of living cultures in feed for various animals in order to substitute the application of nutritive antibiotics or chemotherapeutics [51]. The current defnition formulated in 2002 by FAO and WHO experts defines probiotics as "live strains of strictly selected microorganisms administered in adequate amounts and confer a health benefit on the host" [19]. The definition was in 2013 maintained by the International Scientific Association for Probiotics and Prebiotics (ISAPP). The term "probiotic' is reserved for formulas/products that keep some strictly defined criteria including: an appropriate count of viable cells, a beneficial effect on a host's health involving stimulation of growth, and a beneficial effect on the function of the gastrointestinal tract. Also the selection of bacterial strains with probiotic properties (probiotic cultures) and their application in a correct form as well as dose administered directly per os or as an additive to feed and premixes are highly important [43]. The selection of a suitable strain of a microorganism can be regarded as the primary requirement for the use as a probiotic. These cultures must be able to pass the stomach-duodenum barrier in a viable state and to multiply at the site of destination in the intestine. Additionally, they must be capable of producing antagonistic metabolites against a dominating saprophytic microflora resulting in a competitive growth. These abilities are common among lactic acid bacteria, e.g. lactobacilli bifidobacteria and enterococci. The special efficacy of probiotics must be strictly verified in animal nutrition, in pharmacy, and in food applications in accordance with law regulations. Safety aspects are considered very restrictively in feed applications, replace the presently reduced or even prohibited application of nutritive antibiotics or chemotherapeutics in animal nutrition [51]. Along with the intensive development of methods of livestock breeding, breeders' expectations are growing concerning feed additives that would guarantee such results as accelerating growth rate, protection of health from pathogenic infections and improvement of other production parameters such as: absorption of feed and quality of meat, milk and eggs. The main reason for their application would be to achieve some benefcial effects comparable to those of antibiotic based growth stimulators, banned in the European Union in 2006. High hopes are being associated with the use of probiotics, prebiotics and synbiotics as the alternative natural substances in animal nutrition [43].

In the USA, microorganisms used for consumption purposes should have the Generally Regarded As Safe (GRAS) status, regulated by the US Food and Drug Administration (USFDA). In Europe, the European Food Safety Authority (EFSA) introduced the term of Qualified Presumption of Safety (QPS), which involves some additional criteria of the safety assessment of bacterial supplements, including the history of safe usage and absence of the risk of acquired resistance to antibiotics [21]. In 2006, the EFSA established the Nutrition and Health Claims regulation (Reg. 1924/2006) which was updated by QPS under EFSA's Panel on Biological Hazards during 2008 and 2009. The presence of transmissible antibiotic resistance markers in the evaluation of the strains has been established as the important health criterion. Following these rules, microbes claimed as probiotic in food/feed are supposed to be QPS probiotic (e.g. *Lactobacillus* sp. or *Bacillus* sp.) and non-QPS probiotic (e.g. *Enterococcus faecium*) [50]. According to this assessment, some *Enterococcus faecium* strains can be used for this purpose [33].

Basic properties of potentially probiotic organismS

The major properties of selected probiotic strains include their safety of human/animal origin, the isolation from healthy organism, the detection based on the phenotypization and genotypization, survival in dynamic variations of pH, viable cell counts, the absence of genes responsible for antibiotic resistance (also confirmed plasmid encoded antibiotic resistance) [2], the absence of the production of virulence factors (due to evaluation of genes encoding potential virulence factors e.g. cytolysin cylA, cylB, cylM; collagen-binding protein ace; gelatinize gelE; aggregation substance agg; cell wall anchored collagen adhesion acm; enterococcal surface protein esp) [47], bacterial adhesion to hydrophobic compounds, acid and bile salts tolerance, resistance to enzymes, stimulated gastrointestinal tract tolerance, cell adhesion/hydrophobic characteristics, the ability to colonize and survive in a beneficial dose and competitiveness in part of the intestinal tract, killer toxin productivity and antimicrobial activity against some clinical and food borne pathogens and survivability during simulated gastrointestinal transit. Subspecies were identified by partial 16S rRNA gene sequencing [55, 43, 68]. Probiotic use may help decrease the rate of development of antibiotic-resistant strains secondary to widespread antibiotic use. Given the emerging risk of spreading antibiotic resistance genes through probiotic strains, the qualified presumption of safety (QPS) is considered by many as the more applicable and flexible probiotics criteria [17, 18]. It is important that no other substances are used while probiotics are administered. An interval of 24—48 h between the end of antibiotic therapy or administration of any other antimicrobial agents and the beginning of the therapy with probiotic organisms presented in appropriate amounts 10^9 CFU.kg^{-1} of feed is very important [54]. Probiotics are gaining more interest as alternatives for antibiotics or anti-inflammatory drugs, modulate the host's immune system, affect other microorganisms or act on microbial products, host products or food components. What kind of effect(s) a certain probiotic executes depends on its metabolic properties, the molecules presented at its surface or on the components secreted. Even integral parts of the bacterial cell such as its DNA or peptidoglycan might be of importance for its probiotic effectiveness. The individual combination of properties in a certain probiotic strain determines its specific probiotic action and as a consequence its effective application for the prevention and/or treatment of a certain disease [49].

The resistance of probiotics toward technological processing

Probiotics are available commercially in many forms, including foods, dietary supplements, and clinical therapeutics with oral or non-oral delivery, e.g. lactic acid producing genera such as the bifidobacteria or lactobacilli or enterococci. To be a candidate for commercialization, a probiotic must retain its properties during large-scale industrial preparation and remain stable during storage and use. The probiotic should be able to survive in the intestinal ecosystem and the host animal should gain beneficially from its presence. Clearly, the organisms used should be generally regarded as safe due to USFDA as well as EFSA regulations or well documented in the literature [64].

Probiotic bacteria as probiotics used in technologies of pharmacological industry may be exposed to various environmental stresses during industrial production steps, including drying and storage, and during the digestion process. In accordance with their adaptation as well as survival to environmental conditions, they possess adaptation mechanisms, which can be induced by pre-treatments including the accumulation of compatible solutes and of energy storage compounds, which can be largely modu-

lated by the culture conditions. The regulation of energy production pathways, the modulation of the cell envelope, i.e. membrane, cell wall, surface layers, and exopolysaccharides leads to the overexpression of molecular chaperones and of stress-responsive proteases. Matrix components, such as proteins, carbohydrates and flavouring agents have been shown to alter probiotic efficacy and viability [20].

Many of the effects obtained from viable cells of probiotics are also obtained from populations of dead cells. The probiotic paradox is that both live and dead cells in probiotic products can generate beneficial biological responses. Live probiotic cells influence both the gastrointestinal microflora and the immune response whilst the components of dead cells exert an anti-inflammatory response in the gastrointestinal tract. Heat-killed cells of *Enterococcus faecalis* stimulate the gastrointestinal immune system in chicks. Dead bifidobacteria induce significant increases in TNF-α production. Administration of heat-killed *E. faecalis* to healthy dogs increases neutrophil phagocytes [9].

The heat-killed, ultraviolet-inactivated, and even cell walls microencapsulated components of probiotics may be safer for the host. They finally lead to the overexpression of molecular chaperones and of stress-responsive proteases. Triggering these adaptive mechanisms can improve the resistance of beneficial bacteria toward technological and digestive stresses [22]. In fact, the microencapsulation of probiotics (probiotics in coated protected form) with specific materials is able to confer a significant resistance to gastric juice, thus protecting the probiotic cells during the gastric and duodenal transit and enhancing the probiotic efficacy [12]. Microencapsulation is a process by which individual particles or droplets of solid, liquid or gaseous material (the core; the intrinsic part) are surrounded or coated with a continuous film of material (the shell; the extrinsic part) to produce capsules in the micrometre to millimetre range, known as microcapsules (have a spherical or irregular shape). Compatibility of the core material with the shell is an important criterion for enhancing the efficiency of microencapsulation [23, 65]. There are some techniques which are used for microencapsulation, such as chemical (suspension, dispersion, emulsion, and polymerization); physicochemical (layer by layer assembly, sol gel encapsulation, supercritical CO_2 extraction); physico mechanical (spray drying, fluid bed coating, electrostatic encapsulation) [7]. Microencapsulation has been proven to be one of the most effective methods for maintaining high viability and stability of probiotic bacteria, as it protects probiotics both during food processing and storage as well as in gastric conditions [12, 52]. Chandramouli et al. [26] shoved that the coating of the calcium chloride on sodium alginate capsules containing *L. acidophilus* increased tolerance of the bacteria against harsh acidic (pH 2) and bile (1 %) conditions. The microencapsulation techniques using an alginate microparticulate system potentiality of various coating polymers such as chitosan and polylysine improved the stability of microencapsulation [12, 27].

In conclusion, probiotic cultures added to feed should be resistant to temperatures and pressures used in the process of pelleting, and to humidity and the effect of adverse substances during feed handling and storage, such as heavy metals or mycotoxins. The period of high activity of probiotics in feed and premixes must not be shorter than 4 months [46].

Immunity, intestinal mucosa and probiotics

The mucosal immune system must constantly monitor the environment and maintain a balance between tolerance to the normal microbiota and immunity to microbial pathogens while the systemic immune system is designed to vigorously react to any foreign antigen or microbe [11]. Development of 16S ribosomal RNA (rRNA) gene-sequence-based metagenomic methods has led to major advances in defining the total microbial population of the gut. This technique has been used to show that 90% of the bacteria belong to two phyla, the Bacteroidetes and Firmicutes [64]. The presence of beneficially acting bacteria in the intestine can influence the host and bacterial microenvironment to protect the homeostasis and effective immune response. IgA antibodies belong to the most important humoral immune factors present on mucosal surfaces.

Different defence mechanisms are involved in the permanent and effective surveillance of mucosal surfaces. Bacterial behaviours depend not only on the bacterial species, but also on the host. Commensal bacteria have been directly associated with the proper development of gut-associated lymphoid tissues. Mucosal antibodies inhibit the adherence of microorganisms and protect the host against absorption of antigens from mucosal surfaces [25]. Mucosal surfaces comprise various lymphoid structures collectively referred to as mucosa-associated lymphoid tissue (MALT) [40]. This secondary lymphoid organ can be further divided into functionally connected subregions, including

the gut-associated lymphoid tissue (GALT). A key component of this interface is the mucosal epithelium, which blocks invasion by pathogenic and commensal bacteria by forming multiple layers of immune protection [1] as well as maintaining the host–microbiota relationship in a dynamic homeostasis [53].

Enterocytes have a role not only in the digestion by ensuring the uptake of ions, water, nutrients, vitamins and absorption of unconjugated bile salts, but also in the induction of immunological tolerance to ingested proteins [45]. The epithelial barrier protects the internal medium from food antigens as well as from bacteria. The distal small intestine, caecum and colon have higher bacterial colonization levels than the proximal part. The small intestine contains lower numbers of commensal bacteria and contains higher levels of nutrients available for absorption. The small intestine has higher numbers of intraepithelial T cells; it also harbours lymphoid structures such as Peyer's patches and Paneth cells producing anti-microbial peptides [45]. The intestinal mucus layer is a balance of mucin secretion and degradation. This mucin layer creates an obstacle to proinflammatory compounds and uptake of antigens.

The intestinal lumen consisting of gastric acid, digestive enzymes and IgA constitutes the first line of defence and is lethal to invading and ingested pathogenic bacteria. The indigenous microbes degrade intraluminal antigens and inhibit the pathogenic microbes from adherence and colonization. They are also necessary for the induction of regulatory T cells [64]. The barrier function of the enterocytes is completed by anti-microbial peptides and mucin proteins production [45]. The administered probiotics stimulate the mucosal immune system (MIS) of the intestinal tract and induce signals mediated by the bacteria or their cell wall structure. Consumed probiotic bacteria interact with the intestinal epithelial cells (IEC) or immune cells associated with the lamina propria, through pattern recognition receptors such as Toll-like receptors (induce the production of different cytokines or chemokines) and nucleotide binding oligomerization domain-containing protein-like receptors, which modulate key signalling pathways, such as nuclear factor-κB and mitogen-activated protein kinase [5, 64]. The Toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors play a key role in pathogen recognition and in the induction of innate effectors and inflammation. Pattern recognition receptors signalling in the IEC serve to maintain the barrier functions of the epithelium, including the translocation from the lamina propria in the intestinal lumen and the production of secretory IgA (sIgA). The IEC play a role in the immunosuppressive effect of the mucosa by inhibition of an overreaction against innocuous luminal antigens (due to the regulation of dendritic cells, macrophage and lymphocyte functions by epithelial secreted cytokines) [45, 67].

Macrophage chemoattractant protein 1 (produced by the IEC) sends signals to other immune cells leading to the activation of the MIS, characterized by an increase in immunoglobulin A+ cells of the intestine, and the activation of T cells (specifically activation regulatory T cells that release interleukin IL-10) [41]. Secretory sIgA antibodies at mucosal surfaces serve as the first line of defence against microorganisms through a mechanism called immune exclusion, fight pathogens without the damage of epithelial cells and improve the immune balance of the epithelial barrier through selective adhesion to M cells in intestinal Peyer's patches. In Peyer's patches, sIgA-based immune complexes are internalized by underlying antigen-presenting cells, leaving the antigen with masked epitopes, which translates into the onset of mucosal and systemic responses associated with the production of anti-inflammatory cytokines [10].

In conclusion, probiotics reinforce the intestinal barrier by an increase of the mucins, the tight junction proteins and the Goblet and Paneth cells, modulate intestinal microbiota by maintaining the balance and suppressing the growth of potential pathogenic bacteria in the gut [41].

The effect of the oral administration of probiotic bacteria cell walls as a new oral adjuvant in the stimulation of the immune system in healthy mice on IEC which are essential for coordinating an adequate mucosal immune response and on the functionality of macrophages was evaluated. The cell walls were able to stimulate the IEC exhibiting an important activation and cytokine releases as well as promoted macrophage activation from peritoneum and spleen, improving the functionality of the macrophages and increased IgA-producing cells in the gut lamina propria [37].

Some commensals are able to stimulate local immune response as shown in the case of the application of *Enterococcus faecium* AL41 to chickens infected with *Salmonella Enteritidis*. Immunohistochemical analysis revealed an increased number of IgA+ cells in the caecum after 7 days [6]. Also the effect of probiotic *Enterococcus faecium* AL41

(an environmental isolate) [42] on TGF-ß4 and IL-17 expression and on immunocompetent cell distribution after *Campylobacter jejuni* infection in broiler chickens was observed. The expression of selected cytokines (upregulation of TGF-ß4 but downregulation of IL-17 relative expression), and activation of IgA-producing cells in the caeca of chicks infected with *C. jejuni* CCM6191 was recorded [30, 38]. The immunomodulation effect on inflammatory response was revealed after the exposure of Intestinal porcine epithelial cells with Lactobacillus reuteri B6/1 under in vitro conditions presented by mRNA expression levels analysis of inflammatory cytokines (IL-8, IL-18) and transcriptional factors (MyD88 and NF-κβ) [60].

Probiotics are able to confer health benefits to the host, including specific gastrointestinal effects such as: reduction of the number of pathogens, secretion of enzymes and bacteriocins, improvement of immunomodulation, affection of proliferative activity of intestinal mucosa in various animal ecosystems [35, 36, 39, 56, 57, 58, 61]. The antimicrobial effect was evaluated in the pilot experiment with the application of enterocin M-producing strain *Enterococcus faecium* CCM8558 to infected chickens with *Campylobacter jejuni* CCM6191, while a significant increase in phagocytic activity was also noted in experimentally infected groups treated with the probiotic strain mentioned above [34].

Research has demonstrated that the administration of probiotics to the normal gut microbiota by stimulating the gastrointestinal immune response (antibody production and increasing phagocytic activity) can support the animal's defence systems against invading pathogens [6, 34].

THE role of probiotics in altering THE brain function

The mechanism whereby probiotic ingestion leads to changes in brain function and behaviour involves changes in gut permeability, and shifts in systemic immunity with decreased production of proinflammatory cytokines, including TNF-α [13]. These pathways traditionally have included signalling via neural pathways (mainly vagal nerve afferents) and immune signalling (mainly via circulating cytokines, which either enter the brain directly or activate cerebral endothelium) [8].

A novel peripheral signalling pathway was described occurring in the condition of liver inflammation, which involves increased peripheral TNF-α production driving increased microglial activation, followed by monocyte recruitment into brain vasculature and brain parenchyma, which in turn drives the development of sickness behaviours [14]. The potential peripheral pathways that link probiotic ingestion to changes in the brain function have primarily focused on the role of the changes in cerebral levels of neuromodulators such as brain-derived neurotrophic factor [3, 4].

Probiotic consumption has also been shown to alter brain function and behaviour in healthy organism. Specifically, probiotic ingestion can have beneficial effects on mood and cognition [44] and has also been associated with changes in neural activity in brain regions involved in emotional processing [62]. Changes in cross-talk among the intestinal epithelium, the intestinal immune system, and gut microbes has increasingly been recognized for its capacity to: modulate systemic immunity and prevent peripheral inflammation associated with increases in circulating TNF-α levels, cerebral microglial activation, and recruitment of activated monocytes into the brain. The probiotic therapy may have a therapeutic role in regulating peripheral inflammation-associated brain dysfunction and behavioural alterations [16, 24]. D' M e l l o et al. [15] defined a novel pathway of probiotic mixture VSL#3 (containing eight live, freeze-dried bacterial species: *Streptococcus salivarius* subsp. *thermophilus, Bifidobacterium breve, B. infanti, B. longum, Lactobacillus acidophilus, L. planarum, L. casei,* and *L. delbrueeki* subsp. *bulgaricus*) ingestion that prevented peripheral inflammation. Therefore, probiotic therapy may have a therapeutic role in regulating peripheral inflammation-associated brain dysfunction and behavioural alterations which may affect the the patient's quality of life.

Lactobacillus supplementation is beneficial to the barrier function of the intestinal physical barrier in piglets, e.g. the effects of dietary supplementation with *L. acidophilus* on the performance, intestinal physical barrier functioning, and NOD-like receptors (NLRs) were expressed in weaned piglets. As a result, dietary *L. acidophilus* supplementation was found to increase the average daily gain and reduce the serum diamine oxidase activity. These results demonstrated that *L. acidophilus* supplementation improved the growth performance, enhanced the intestinal physical barrier function, and inhibited the expression of NOD1 and NLR family pyrin domain containing 3 (NLRP3) signaling-pathway-related genes in the jejunum and ileum tissues, enhances the intestinal physical barrier functioning by inhibiting interleukin IL-1β and IL-18 proinflammatory cytokines via the NOD1/NLRP3 signalling pathway in weaned piglets [66].

The gut-brain-microbiota axis is increasingly recognized as an important regulator of intestinal physiology. Exposure to psychological stress causes activation of the hypothalamic-pituitary-adrenal (HPA) axis and causes altered intestinal barrier function, intestinal dysbiosis, and behavioural changes. The effects of psychological stress on intestinal physiology and behaviour, including anxiety and memory were investigated in mice. Both local (intestinal physiology and microbiota) and central (behavioural and hippocampal decreased c-Fos expression) changes were normalized by pre-treatment with probiotics, indicating an overall benefit on health conferred by changes in the microbiota. These findings indicate and show that probiotics can overcome this immune-mediated deficit in the gut-brain-microbiota axis [55]. Joseph and Law [29] conducted a cross-species examination of single- and multi-strain combinations of established probiotics while 58 non-human (twenty-five rat, twenty-seven mouse, five zebrafish, one quail) investigations satisfied the criteria. For the non-human studies, single- (60.5 %) and multi-strain (45.0 %) combinations modified stress, anxiety, or depression behaviours in addition to altering social or cognitive performance (single-strain 57.9 %; multi-strain 85.0 %).

The application of probiotic microorganisms can be considered as a suitable alternative to antibiotics as well as representing a way to effectively influence the composition of the intestinal microbiome and the immune system of the host. On the other hand, the other possibility of using probiotics is the influencing of the connection between the intestine and the brain through the gut-brain axis. The further studies of the presented problem related to alternative use of probiotics is needed.

ACKNOWLEDGEMENT

This study was supported by the project VEGA No. 1/0658/17.

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Received August 27, 2019
Accepted October 14, 2019