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Roles of Probiotic Lactobacilli Inclusion in Helping Piglets Establish Healthy Intestinal Inter-environment for Pathogen Defense

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Abstract The gastrointestinal tract of pigs is densely populated with microorganisms that closely interact with the host and with ingested feed. Gut microbiota benefits the host by providing nutrients from dietary substrates and modulating the development and function of the digestive and immune systems. An optimized gastrointestinal microbiome is crucial for pigs' health, and establishment of the microbiome in piglets is especially important for growth and disease resistance. However, the microbiome in the gastrointestinal tract of piglets is immature and easily influenced by the environment. Supplementing the microbiome of piglets with probiotic bacteria such as *Lactobacillus* could help create an optimized microbiome by improving the abundance and number of lactobacilli and other indigenous probiotic bacteria. Dominant indigenous probiotic bacteria could improve piglets' growth and immunity through certain cascade signal transduction pathways. The piglet body provides a permissive habitat and nutrients for bacterial colonization and growth. In return, probiotic bacteria produce prebiotics such as short-chain fatty acids and bacteriocins that benefit piglets by enhancing their growth and reducing their risk of enteric infection by pathogens. A comprehensive understanding of the interactions between piglets and members of their gut microbiota will help develop new dietary interventions that can enhance piglets' growth, protect piglets from enteric diseases caused by pathogenic bacteria, and maximize host feed utilization.

Keywords Lactobacillus · Microbiome · Piglets · Pathogen · Defense

Introduction

Microbiota in the gastrointestinal (GI) tract is an important environmental factor for health [1], because it has evolutionarily conserved roles in the metabolism, immunity, development, and behavior of the host [2, 3]. There is huge diversity in the bacterial species that constitute the intestinal microbiota: approximately 160 species of bacteria are present within every individual [4]. Considerable efforts have focused on cataloging the human gut microbiome and its relationship to complex diseases [5–7]. Many of these studies have been conducted in model organisms. Societal pressure to reduce the number of non-human primates and dogs used in biomedical research has led to an increase in the use of pigs. Pigs are easy to keep and collecting samples from pigs is simple. In addition, the anatomy and physiology of pigs have notable similarities to humans. These factors have helped to establish pigs as a model organism in research fields [8]. However, studies on the gut microbiota of pigs, and especially piglets, have been limited. In addition, factors that affect establishment of intervention on the gut microbiota in piglets and its relationship to pathogen defense have not been satisfactorily reported. The objective of this review was to evaluate the effect and potential mechanism of probiotic *Lactobacillus* supplementation in piglets and to

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provide the basis for supplementation of probiotic bacteria in human infants.

Probiotics contain live microorganisms and spores, and when administered in adequate amounts, confer health benefits to the host [9, 10]. The benefits of probiotic application in humans and animals include inhibition of pathogens [11, 12], improved digestive function [13], and modulation of immune responses [14, 15]. In animal agriculture, probiotics are thought to be an important potential alternative to the use of antibiotic growth promoters (AGP) [16, 17]. When used in piglets, probiotics have been demonstrated to promote growth performance at levels similar to AGPs [18] and to reduce gastrointestinal colonization by pathogens [19].

Lactobacillus

Lactobacillus species are common probiotics, and they play important roles in pathogen defense and improved immunity in piglets [20–22]. The number of described species in the genus *Lactobacillus* has increased considerably during the last 10–15 years. There are currently 151 described *Lactobacillus* species [23]. A recent study [24] using next-generation sequencing technology identified *Lactobacillus* as a core fecal microbiota across the growth stages of pigs. Swine fecal microbiota was found to change significantly across growth stages, but populations of the core members were stable. Furthermore, Niu et al. [25] found that *Lactobacillus* is one of the most dominant genera, accounting for approximately 15% of 16S rRNA gene sequences from porcine intestinal samples, regardless of age. In addition, the most important end product of fermentation by lactobacilli is lactic acid, which is crucial for piglets' health.

Inhibition of Pathogens

In order to cause infections in piglets, enteric pathogens need to first attach to and then breach the intestinal epithelial barrier [26]. In healthy piglets, commensal bacterial communities in the GI tract colonize intestinal mucosa and form a layer of bacteria covering the mucosal surface. By occupying a diverse array of adherence niches along the GI tract, this layer of dense and complex microbial communities can effectively block the attachment and subsequent colonization by most invading enteric pathogens [27]. This phenomenon is called competitive exclusion [28]. The ability of probiotic lactobacilli to reduce colonization of bacterial pathogens in the gastrointestinal tract is very important for piglets' health. Probiotic lactobacilli exclude pathogens from attaching to mucosal surfaces through competition for shared binding sites [29, 30] and steric interference of protein adhesins located on the surface of pathogenic bacteria [31, 32]. In addition to preventing adhesion, an in vitro study demonstrated the varying ability of *Lactobacillus (L.) acidophilus* TMC 0356 and *Lactobacillus rhamnosus* TMC 0503 to displace *Salmonella typhimurium*, *Cronobacter sakazakii*, *Clostridium difficile*, and *Escherichia coli* which were already adherent to human epithelial cells [33]. Inhibition of pathogen adherence is also seen in a pig intestinal mucosa model [34]. These studies demonstrate probiotic strain and host-specific inhibition of pathogens, highlighting the need for case-by-case selection of probiotic cultures to reduce adherence of specific pathogens. The production of pathogen-inhibiting compounds is a well understood probiotic mechanism [35]. Neal-McKinney et al. [36] demonstrated that the production of lactic acid by *Lactobacillus* cultures is an important mechanism for the reduction of *Campylobacter jejuni* in livestock animals. Hydrogen peroxide production by *Lactobacillus* has also been shown to inhibit *Salmonella* [37].

Enhancement of Barrier Function

The intestinal epithelium is specialized to ensure optimal absorption of nutritional compounds, yet at the same time to exclude and neutralize or detoxify harmful components of the intestinal contents including microorganisms. In a healthy gut these functions are optimized and a healthy epithelium is essential to maintaining a healthy gut [38]. The epithelial lining consists of a single layer of epithelial cells covered by layers of mucus produced by specialized goblet cells. Epithelial cells are joined together by cell junctions such as tight junctions (TJs). TJs play a major role in preventing molecules from entering the epithelium between cells [39]. Various stressors may cause weakening of TJs and increase un-regulated paracellular transport of macromolecules into the mucosa. The uncontrolled diffusion of intraluminal toxins, antigens, and enteric microbiota to the underlying tissue results in local and systemic inflammation. The potential of lactobacilli to strengthen the epithelial barrier can be evaluated in vitro by determining the trans-epithelial electrical resistance (TER) in epithelial monolayer cell lines such as Caco-2 or HT29 epithelial cells. TER is dependent on the paracellular flux of ions which is regulated primarily by TJs. Stimulation of TJs may be caused by short-chain fatty acids produced by lactobacilli [40]. Fermentation products of lactobacilli from various types of prebiotic carbohydrates increased TER in Caco-2 monolayers, and the effect was strain and prebiotic dependent.

Improvement of barrier function by lactobacilli and the consequences for disease have been demonstrated in animal models [41]. Stress, especially in the early weaning stage of piglets, induces changes in gut microbiota and the gut epithelial barrier [42, 43]. Previous studies have indicated that stress greatly affects the gastrointestinal microflora, decreasing total *Lactobacillus* populations in severely stressed animals, and thus providing an opportunity for overgrowth of pathogens [44]. In support of this, Bateup et al. [45] found that the composition of *Lactobacillus* populations, especially in the stomach and caecal contents of 24-day-old pigs, showed evidence of instability during this stressful period.

Improvement of Immunity

The immune system of the intestinal tract, referred to as the gut-associated lymphoid tissues (GALT), contains the largest pool of immunocompetent cells in the human body [46]. The major function of the GALT is to control our relationship with the microbiota. A central strategy is to minimize contact between microorganisms and the epithelial cell surface, thereby limiting tissue inflammation and microbial translocation [47]. Commensal microorganisms that penetrate the epithelial barrier will be rapidly phagocytosed and destroyed by intestinal macrophages. This dialogue between the gut microbiota and the immune system allows the host to tolerate a large amount of antigens in the gut. Intestinal bacteria at the mucosal surface can create signals called microbial-associated molecular patterns (MAMPs) that stimulate pattern recognition receptors. For example, toll-like receptors (TLR), expressed on the surface of epithelial cells, trigger a cascade of immunological defense mechanisms including the production of antimicrobial peptides, pro- and anti-inflammatory cytokines, or triggers for apoptosis [48]. In turn, to protect their ecological niche, a dominant action of the healthy microbiota on the immune system is to reinforce barrier immunity and therefore their own containment.

Most of probiotic effector molecules are present in the bacterial cell envelope, which is the first side to interact with host intestinal cells [49, 50]. In lactobacilli the cytoplasmic membrane is covered by pentapeptide stem-connected layers of peptidoglycan, which has been shown to modulate immune responses [51, 52]. This layer also serves as a platform for anchoring cell surface molecules, such as wall teichoic acids, wall polysaccharides, and surface proteins [53–55]. In lactobacilli, the disaccharide unit of peptidoglycan can undergo a wide range of modifications, which have important consequences for bacterial physiology. In addition to peptidoglycan, all lactobacilli produce lipoteichoic acid, which contains di-acylated and/or tri-acylated glycolipids [56, 57] that are thought to signal via the heterodimeric TLR complexes TLR-2/6 and TLR-2/1, respectively [58].

In addition to cell wall associated molecules, bacterial genomic DNA can also interact with the host. TLR-9 recognizes bacterial genomic DNA, which, unlike eukaryotic DNA, contains a high frequency of unmethylated CpG motifs [59]. Different species of lactic acid bacteria might differ in their capacity to elicit TLR-9 signaling due to differences in C+G composition and the frequency of stimulatory motifs in the DNA. The expression of TLR-9 by immune cells is intracellular and endosomal, and in polarized epithelial cells, it is expressed on both the apical and basolateral membranes. In polarized epithelial cells, TLR-9 has been shown to have tolerogenic effects to chronic TLR challenges depending on the location of the stimulus [60].

Considering the immature microbiota composition in the GI tract and the inadequate immune system of piglets, supplementation with lactobacilli could effectively activate the body to establish immunity to defend against infection of pathogens.

Modification of the Microbiota Composition

Probiotic lactobacilli can be used in piglets to support the development of a stable microbiota, to prevent diarrheal diseases. During the weaning and post-weaning periods, lactobacilli are used in pigs to modulate the gastrointestinal microbiota to prevent post-weaning diarrhea and stimulate growth. Yang et al. [61] showed that *Lactobacillus plantarum* significantly decreased *E. coli* and aerobe counts and increased lactobacilli and anaerobe counts in the digesta and mucosa of most sections of the GI tract compared with a control group. Liu et al. [62] reported that *Lactobacillus reuteri* I5007 plays a positive role in gut development in piglets by modulating microbial composition and intestinal development. Denaturing gradient gel electrophoresis revealed that *L. reuteri* I5007 affected the colonic microbial communities on day 14, in particular, and reduced numbers of *Clostridium* spp. In weaning pigs, administration of *L. reuteri* BSA131 decreased the number of enterobacteria in the feces [63]. The mechanism that contributes to the selective stimulation of bacterial groups by lactobacilli supplementation relates to cross-feeding. Cross-feeding is the phenomenon of partial degradation products being released by primary degraders that stimulate the growth of other bacterial groups. For instance, lactate produced by lactobacilli can be converted by lactate-utilizing bacteria, such as *Eubacterium* and *Anaerostipes*, to produce butyrate [64]. Other butyrate-producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia* spp., mostly belong to the *Firmicutes* phylum and convert acetate into butyrate [65, 66]. A stimulation of *F. prausnitzii* was observed after dietary intervention with inulin (10 g/day for 16 days) in healthy subjects [67]. This mechanism also explains the butyrogenic effect observed after *Lactobacillus* administration. Lactobacilli do not produce butyrate but provide lactate or acetate for cross-feeding to those other bacterial groups.

Function of Short-Chain Fatty Acids

Short-chain fatty acids (SCFA) are the major anions within the intestinal lumen and are mainly produced by anaerobic fermentation of undigested carbohydrates and, to a lesser extent, proteins [68, 69]. Most of the SCFA formed by intestinal bacteria are rapidly absorbed and used to some degree as energy substrates by mucosal epithelial cells. In this way, SCFA provide about 10% of the daily caloric requirements in humans [70], with butyrate being the preferred energy source for the colonocytes. Readily fermentable dietary fiber has been shown to stimulate epithelial cell proliferation in the intestine only in the presence of gut bacteria, suggesting that the end products of fermentation are responsible for this effect [71]. Increased SCFA synthesis also contributes to host homeostasis by acidifying the luminal pH, which inhibits the growth of pathogens [72], reduces the formation of secondary bile acids [73], and impairs the activity of specific enzymes such as proteases. Furthermore, SCFA have been shown to possess anti-inflammatory capacities, affect satiety hormones, and play a role in insulin resistance [74]. SCFA are speculated to have a role in prevention of some human pathological conditions such as ulcerative colitis and colon carcinogenesis. Diversion colitis, which occurs in diverted segments of the large bowel excluded from fecal transit, improves after treatment with a local perfusion of SCFA [75].

Many health benefits in and outside the gut have been attributed to increased production of SCFA by stimulated beneficial bacteria. Simple acidification of the colonic lumen by the production of SCFA can explain some of the observed benefits of prebiotics. In addition, SCFA are considered as a class of bacterial products that mediate the interactions between the diet, the intestinal microbiota, and the host. Two major SCFA signaling mechanisms have been identified: the inhibition of histone deacetylase and the activation of G-protein coupled receptors [76]. In addition, SCFA easily enter the cells through passive diffusion or receptor-mediated transport and can internally act at other sites [77, 78]. The transduction pathway of butyrate-induced apoptosis has been shown to involve the activation of the caspase cascade. Butyrate activates p38 mitogen-activated protein kinase (p38 MAPK), which in turn up-regulates expression and receptor activity of the peroxisome proliferator activated receptor gamma (PPARγ). PPARγ activates caspase-8 and caspase-9 leading to increased caspase-3 activity which will eventually result in cell death [79]. Activation of PPARγ has been effective in the prophylaxis, and to a lesser extent, in the treatment of several animal models of acute or chronic colitis [80, 81]. PPARγ plays a fundamentally important role in the immune response through its ability to inhibit the expression of inflammatory cytokines and to direct the differentiation of immune cells towards anti-inflammatory phenotypes [82, 83].

Protective Function of Other Metabolites from Lactobacilli

The intestinal mucosa is the interface between the internal and external environments and forms a crucial line of defense to prevent luminal pathogens and harmful substances from entering into the internal milieu. This barrier function is ensured by protection mechanisms at multiple levels [84]. Certain probiotic lactobacilli have the ability to secrete antimicrobial substances, such as bacteriocins and organic acids, which inhibit the growth of other bacteria. The production of bacteriocins by probiotic lactobacilli has the potential to prevent gastrointestinal infection in humans. A direct challenge study in mice demonstrated that bacteriocin production by *Lactobacillus salivarius* UCC118 reduced counts of *Listeria monocytogenes* by 80% in the liver and spleen of infected mice relative to a negative control [85]. *L. salivarius* UCC118 also protected mice from infection by *S typhimurium*, but the protection was not bacteriocin mediated. While bacteriocin production by probiotic cultures is hoped to be an important alternative to antibiotics in the treatment of bacterial infections, the effectiveness of this mechanism has not yet been evaluated in humans.

Supplementation of *lactobacilli* results in a decrease of the colonic luminal pH due to the production of lacto acid, which affects the composition of the microbiota due to the differential sensitivity of bacterial species to acidic pH. *Bacteroides* spp. are relatively sensitive to mildly acidic pH, whereas Firmicutes spp. and bifidobacteria are more acid tolerant and are therefore less affected by a decrease in pH [86]. Because of special physiological characteristics, piglet guts lack enough acid to help food metabolism. Hence, supplementation with lactobacilli could effectively promote metabolism and nutrition absorption.

The secretion of mucus and immunoglobulin A by different epithelial cells minimizes the chances for direct contact of bacteria with epithelial cells. Commensal species have been shown to limit pathogen colonization through competition for nutrients and adhesion sites, a process called colonization resistance [87]. *Lactobacillus GG* could prevent cytokine-induced apoptosis and inhibit pro-apoptotic p38/MAPK activation [88, 89]. These factors are also able to modulate hydrogen peroxide induced damage in Caco-2 cells [90]. Other molecules produced by lactobacilli have been found to have important characteristics. An analysis of genomic sequences from *Lactobacillus* strains predicts a broad group of bacteriocins that are active against Gram-positive bacteria such as *L. salivarius* UCC118. A class II bacteriocin produced by *Lactobacillus* strains also has the ability to protect mice against infection with *L. monocytogenes* [85]. Several other lactobacilli have been tested in different in vivo and in vitro tests with positive results [91, 92].

Need for Caution

Because of special physiological characteristics of piglets such as a scarcity of acids secreted by the stomach, as well as immature microbiomes and immune systems, supplementation of lactobacilli could effectively enhance the GI tract environment by inducing an optimal composition of microbiota, improving intestinal barrier function, and improving immunity for defense against pathogen infection. However, the results of feeding *Lactobacillus* spp. to pigs are inconsistent, with some reports showing no difference in growth performance of weaning pigs fed diets with and without lactobacilli [93]. It is known that lactobacilli have strain-specific characteristics, and that these may affect specific interactions between bacterial populations and the host. First, the strain was non-indigenous to the farm and may not have possessed the ecological attributes necessary for long-term association with these pigs. Second, the strain was initially predominant in the GI of piglets and may not have been suitably adapted for life in the gastrointestinal milieu of older pigs. Hence, when isolating probiotic bacteria, we should consider the animal species. The probiotic bacteria isolated from an animal species should be use in the certain species in application of the probiotic production. Also, the probiotic bacteria isolated from animals in certain growth period should used in such growth period of animal, which could play efficient roles.

The microbiome in the GI tract of piglets is immature, especially in neonatal animals. Providing probiotic bacteria to neonatal animals to establish a healthy gastrointestinal microbiome would be helpful [8]. Supplementation of probiotic bacteria for animals with disorders of the GI tract occurred caused by stress or pathogen infection also could be important, to reconstitute healthy microbiomes. However, the doses for supplementation are unknown and require further research.

For special human group such as premature infants and hypoimmunity persons, usage of probiotic product needs attention for their adverse reactions, for generalized infection, excessive immune stimulation, gene transfer, or untoward effect of gastrointestinal [94, 95].

Future Prospects

Other strains of probiotics also have been used in neonatal animals including species of *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *Saccharomyces*, *Aspergillus*, and *Bacillus*. *Bifidobacterium* is the predominant genus of the gut microbiota of infants [96]. Owing to their recognized benefits to human health, bifidobacteria also play an important role in the health of neonatal piglets. Therefore, combined use of multiple strains of probiotic bacteria may lead to larger improvements compared to single strains [97].

Considering the beneficial effects of prebiotics such as oligosaccharides, active peptides, and other biologically essential microelements, combined use of probiotic bacteria and prebiotics in piglets may enhance the useful effects. Combined use of organic Chromium and probiotic *Bacillus subtilis* KT260179 might have a greater effect on regulating animal model mouse body metabolism [98]. Use of probiotic cheese, flaxseed, and other prebiotics is a good dietary supplement for piglets before weaning, helping them to adapt to changes in diet more easily and reducing the likelihood of chronic diseases [99, 100].

Culture-dependent methods combined with meta-omics approaches can link proteins and metabolic pathways to functions and probiotic properties of selected strains, which consist of the application of high-throughput culture conditions to the study of the body microbiota and uses matrix-assisted laser desorption/ionization–time of flight or 16S rRNA amplification and sequencing for the identification of growing colonies [101]. Culturomics revolutionized the understanding of the relationships among the human microbiome, health, and diseases and generated a number of sequences that can be assigned to a known microorganism.

Conclusion

The production of piglets has entered an era when the use of antibiotics is increasingly banned. Probiotics, which are a potential alternative to in feed antibiotics, can expect a promising future. Besides, the selection of excellent strains and improved processing techniques, more research, especially in the form of well-designed animal trials, is needed to evaluate the efficacy. More studies are also needed to explore the mechanisms of action of lactobacilli in piglets. With evolving knowledge, effective use of lactobacilli will be possible in the future.

GI, gastrointestinal; TJs, tight junctions; TER, transepithelial electrical resistance; GALT, gut-associated lymphoid tissues; MAMPs, microbial-associated molecular patterns; TLR, toll-like receptors; SCFA, short-chain fatty acids.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate Not applicable.

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