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Probiotics

Current Knowledge and Future Prospects

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PROBIOTICS - CURRENT KNOWLEDGE AND FUTURE PROSPECTS

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Meet the editor

Dr. Shymaa Enany is an assistant professor of Microbiology and Immunology at the Suez Canal University, Egypt. She received her PhD degree from the Graduate School of Medical and Dental Sciences, Niigata University, Japan, and completed her postdoctoral work in collaboration with many laboratories in San Diego, California, USA, and in Niigata, Japan. She is an editorial board member and reviewer in many journals and scientific associations and has many publications in eminent journals as well as books. She has vast experience in bacterial genomics and proteomics.

Contents

Preface

The quality of our life is linked to our daily diet from food that is considered essential and indispensable to human life. Probiotic foods are a group of functional foods that have been used for centuries especially in fermented dairy products since Metchnikoff associated the intake of fermented milk with prolonged life. Probiotics confer many health benefits to humans, animals, and plants when administered in proper amounts. Several recognized profits of probiotics have been proven including the prevention of gastrointestinal infections and antibiotic-associated diarrhea, the reduction of serum cholesterol and allergenic and atopic complaints, and the protection of the immune system. Furthermore, the proper usage of probiotics could suppress *Helicobacter pylori* infection and Crohn's disease, improve inflammatory bowel disease, and prevent cancer.

Many different microorganisms could exert these beneficial effects, and several studies showed the selection methods of strains with high probiotic effect and the development of technologies for the production of improved probiotics. In addition to that, they have been focused on the benefits of the combination of probiotic bacterial strains and prebiotics in functional foods.

We decided to write this book to discuss the different types of probiotic microorganisms and the uses of probiotics and their applications as presented by international leaders in their respective fields.

This book consists of several review chapters. Each chapter starts with a brief introduction, including its aims, and then goes on to provide detailed information about the current research relevant to the field. The authors give an overview of probiotics that they used in their research as important microorganisms that exert beneficial effects on humans and/or animals in a simple way that allows the readers to form a complete picture about these beneficial microorganisms and their suitability as therapeutic and prophylactic agents. Through these chapters, the authors explored the concept of probiotics and prebiotics and how the selection of probiotic microorganisms is done. They examined the beneficial effects of probiotics and their different applications. Future aspects in the development of technologies for the production of improved probiotics are also reviewed here.

We believe that our book is an excellent one for scientists, especially those who are interested in probiotics. We hope you enjoy reading it. Finally, we would like to thank all the contributing authors, without whose dedication and brilliant research, this project would not have been accomplished.

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Chapter 1

Antimicrobial Effects of Probiotics and Novel Probiotic-Based Approaches for Infectious Diseases

Ping Li and Qing Gu

Additional information is available at the end of the chapter

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Abstract

Probiotics are live microorganisms, which confer health benefits on host when administered in adequate amounts. Probiotics exert their beneficial effects by maintenance flora healthy, enhancement of mucosal barrier integrity and modulation of immune responses. Antimicrobial substances including bacteriocins, hydrogen peroxide, organic acids, and short-chain fatty acids (SCFAs) produced by probiotics allow them to inhibit mucosal and epithelial adherence of pathogens and compete for limiting resources, thus suppress the growth of bacterial and fungal pathogens. Probiotics effect the colonization of fungal pathogen *Candida* to host surfaces, suppress *Candida* growth and biofilm development *in vitro*. Clinical results have shown that some probiotics can reduce oral, vaginal, and enteric colonization of *Candida*, alleviate clinical signs and symptoms, and potentially reduce the incidence of invasive fungal infection. Therefore, probiotics may be potential antifungals for prevention and treatment of candidiasis.

Keywords: probiotics, mechanism of action, antimicrobial activity, candidiasis, safety

1. Introduction

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host," which was defined by the Food and Drug Organization of the United Nations (FAO) and World Health Organization (WHO) [1–3]. Probiotics should have some fundamental characteristics, such as human origin, nonpathogenic in nature, resistance to destruction by technical processing, acid and bile tolerances, adequate adherence and colonization on epithelial surfaces, antagonistic activity against pathogens, regulation of immune response, and influence human metabolic activities [4–7].

Bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* are the most frequently used probiotics. Besides, *Enterococcus*, *Streptococcus*, *Saccharomyces*, and *Bacillus* are also commonly used probiotics (representative species are listed in **Table 1**). The administration of probiotics has been confirmed as an alternative biological approach to combat bacterial and fungal pathogens in the oral cavity, GI tract, and urogenital system [4, 5, 7–14]. It has been reported that probiotics could reduce *Candida*, which cause fungal infections in different organ systems of the human body and prevent bacterial infectious diseases [9, 10, 15]. Probiotics were capable of preventing cancers [16], modulating blood pressure [17, 18], and repressing cholesterol levels [19]. Recently, species of *Akkermansia muciniphila*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii* are identified as new potential probiotics because of their great benefits to the microbial metabolic networks and human health, especially the effects on correcting the imbalance of gut microbiota composition [7, 20–22]. A combination of probiotics with traditional treatment has been thought to be a potential approach for treatment of certain diseases.

It is noteworthy that health benefits of probiotic bacteria are strain specific, which cannot be generalized to other strains, not even the same species, although some properties may be common for different strains because of the similarities in the metabolism of ecological functionality [5, 6]. Thus, the selection of certain probiotics for therapeutic purposes should be targeted for specific pathogens. Probiotics effects are dose specific [5, 6]. It has been suggested that a daily intake of 10^6–10^9 colony-forming units (CFUs) of probiotic microorganisms is the minimum effective dose for therapeutic purposes [5, 6, 8].

A number of probiotics are currently commercially available, and they have been categorized into single-strain or multi-strain/multispecies products [7, 23, 24]. Multi-strain/multispecies probiotics exhibited better effects than single-strain probiotics. The multispecies probiotic consortium VSL#3 (*Streptococcus thermophilus, Eubacterium faecium, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus*

Genera	Species
Lactobacillus	*Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus sporogenes*, *Lactobacillus lactis*, *Lactobacillus helveticus*, and *Lactobacillus fermentium*
Lactococcus	*Lactococcus lactis*, *Lactococcus lactis* subsp. lactis, *Lactococcus lactis* subsp. diacetylactis, and *Lactococcus Lactis* subsp. cremoris
Bifidobacterium	*Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium bifidus*, and *Bifidobacterium lactis*
Enterococcus	*Enterococcus faecalis* and *Enterococcus faecium*
Saccharomyces	*Saccharomyces cerevisiae* and *Saccharomyces boulardii*
Streptococcus	*Streptococcus thermophiles*
Bacillus	*Bacillus coagulans* and *Bacillus subtilis*
Others	*Akkermansia muciniphila*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*

Table 1. Representative microbe commonly considered as probiotics.

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plantarum, Lactobacillus casei, and *Lactobacillus delbrueckii* subsp. bulgaricus) was proven more effective than single-strain probiotics for the treatment of ulcerative colitis [23]. The multispecies probiotic consortium, Ecologic AAD (*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W18, *Bifidobacterium longum* W51, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W37 and W55, *Lactobacillus paracasei* W72, *Lactobacillus plantarum* W62, *Lactobacillus rhamnosus* W71, and *Lactobacillus salivarius* W24), combined with amoxicillin, could reduce diarrhea-like bowel movements, while the single strain could not [25]. Thus, the combination-specific probiotic effects from diverse strains can lead to synergistic effects.

Among the most frequently used probiotics, the genera *Lactobacillus, Bifidobacterium, Lactococcus*, and *Saccharomyces* have been included in the category of "generally regarded as safe" (GRAS) [4, 6]; however, other probiotic organisms such as *Enterococcus, Bacillus*, and *Streptococcus* are not generally regarded as safe. Since probiotics have been applied in food production, disease treatment, and others, it is important to undergo safety evaluation of probiotics before human consumption.

In this chapter, we briefly review the mechanisms of action of probiotics, the safety concern of probiotics, and their potentials for prevention and treatment of diseases. Here, we discuss the application of probiotics in the fungal *Candida*-infected and invasion candidiasis.

2. Probiotics mechanism of action

Probiotics mechanism of action is with important differences among different species and strain, examples are listed in **Table 2**.

2.1. Maintenance flora healthy by reduction the growth and colonization of pathogens

The ability of probiotics to establish in the gastrointestinal (GI) tract, maintain flora healthy, and reduce the growth of pathogens and colonization is enhanced by their ability to eliminate competitors. Probiotic strains release different antimicrobial molecules such as organic acids, hydrogen peroxide (H_2O_2), and antimicrobial peptide bacteriocins into the intestinal environment to limit the growth of bacterial and fungal pathogens [6, 39–43].

Lactic acid and acetic acid are the main metabolites formed by lactic acid bacteria (LAB). Both lactic acid and acetic acid could result in acidity environment and thus inhibit the growth of various microorganisms. Acetic acid has a broader spectrum of antimicrobial activity when compared to lactic acid. Moreover, it is known that a synergistic effect exists between the two acids: mixtures of acetic and lactic acids suppress the growth of the pathogenic enteric bacterium *Salmonella typhimurium* [44].

LAB can also produce H_2O_2, the antimicrobial activity of which is linked to the strong oxidizing effect. Hydrogen peroxide showed a bactericidal effect on most pathogens when in

Mechanism of action	Probiotics	Study outcomes	References
Maintenance flora healthy by reduction the growth and colonization of pathogens	*L. rhamnosus* GG, *L. casei* Shirota, *L. reuteri* SD2112 and *L. brevis* CD2	*L. rhamnosus* GG showed the strongest inhibitory activity in fructose and glucose medium against *C. albicans*, followed by *L. casei* Shirota, *L. reuteri* SD2112 and *L. brevis* CD2	[26]
	L. plantarum, commercial preparation LactoLevure®	Increased survival of mice infected by multidrug resistant *P. aeruginosa* and *E. coli*	[27]
	B. breve, *L. casei* (randomized controlled trial, RCT)	Levels of beneficial organic acids significantly increased in the gut, and the incidences of infectious (pneumonia and bacteremia) complications were significantly lower in the probiotic group	[32]
	Synbiotic (*Lactobacillus, Bifidobacterium*, and galactooligosaccharides) for 8 weeks (RCT)	Acetic acid concentration significantly increased (100 times), pH value decreased, Gram-negative rod (1/10) in the gut decreased, and *P. aeruginosa* decreased in the probiotic group	[33]
	Multi-strain synbiotic for 7 days (RCT)	Synbiotic group had lower pathogenic bacteria (43% versus 75%) and multiple organisms (39% versus 75%) in nasogastric aspirates than controls	[34]
	B. lactis Bb12 for 7–21 days (RCT)	Probiotic group had great higher counts of *Bifidobacterium* ($P = 0.001$) and lower counts of *Enterobacteriaceae* ($P = 0.015$) and *Clostridium* spp. ($P = 0.014$) than in placebo group	[35]
	L. casei subsp. *rhamnosus* for 6 weeks (RCT)	Colonization of *Candida* in gut was reduced in probiotic group ($P = 0.01$)	[28]
Enhancement of mucosal barrier integrity	*L. plantarum* 299v for 8 days (RCT)	Bacterial translocation in mesenteric lymph nodes and liver was reduced to 0 and 12%, respectively	[29]
	Microencapsulated *Bifidobacteria*	Bacterial translocation to mesenteric lymph nodes was reduced by encapsulated *Bifidobacteria* ($P < 0.05$)	[30]
	VSL#3 (RCT)	Decreased incidence of bacterial translocation in VSL#3 group than in water group (8% versus 50%; $P = 0.03$)	[31]
Immune modulation	VSL#3 (*Lactobacillus*, *Bifidobacterium*, and *S. thermophilus*) for 7 days (RCT)	Reduced acute physiology and chronic health evaluation II score; reduced sequential organ failure assessment, IL-6, procalcitonin, and protein	[36]
	L. plantarum 299v (RCT)	Late attenuating effect (after 15 days), serum IL-6 levels reduced	[37]

Table 2. Mechanism of action of probiotics.

combination with lactoperoxidase-thiocyanate milk system [45]. *L. johnsonii* NCC933 and *L. gasseri* KS120.1 killed enteric uropathogenic and vaginosis-associated pathogens due to the production of lactic acid and hydrogen peroxide [46].

Bacteriocins are ribosomally synthesized antimicrobial peptides, which have broad spectrum of inhibitory effect against Gram-positive and Gram-negative bacteria, viruses, and fungi [47–50]. *L. plantarum* 2.9, a bacteriocinogenic strain, inhibited a set of foodborne pathogens including *B. cereus*, *E. coli* O157:H7, and *S. enterica* [51]. Bacteriocin-producing strains identified in our lab, e.g., *L. plantarum* ZJ316, *L. plantarum* LZ95, *L. plantarum* ZJ008, and *L. plantarum* ZJ005, showed antimicrobial activity against various pathogens *in vitro* such as *S. aureus*, *E. coli*, *S. enterica*, *L. monocytogenes*, and *C. albicans* [42, 52–54].

2.2. Enhancement of mucosal barrier integrity

Probiotics have been shown to improve barrier function and the mechanisms of barrier function including alteration of tight junction protein expression and/or localization, induction of mucus secretion, increased production of cytoprotective molecules such as heat-shock proteins, inhibition of apoptosis of epithelial cells, and promoting cell survival [29, 55, 56]. They compete with pathogens and prevent their invasion through the epithelium by the ability of adherence to the intestinal epithelium and mucus. *L. plantarum* has been shown to enhance mucosal barrier by adhering to the mucosal membrane and reducing Gram-negative bacteria [29]. Probiotics also compete for limiting resources, thus suppressing the growth of bacterial and fungal pathogens. The probiotic *E. coli* Nissle 1917 is able to effectively take up multiple limited environmental irons and simultaneously competitively inhibit the growth of other intestinal microbes and pathogens [57].

Furthermore, butyrate, a short-chain fatty acid (SCFA), could reduce bacterial translocation, improve the organization of tight junctions, modulate intestinal motility in addition to being an energy source for colonocytes, and maintain the integrity of the intestinal epithelium [29–31, 58–60]. *E. hallii* is an important anaerobic butyrate producer resident in our gut, which influences the intestinal metabolic balance and enhances the host-gut microbiota homeostasis [61]. Thus, the administration of probiotics with butyrate-producing bacteria, in particular, could be an effective way to achieve health benefits.

2.3. Immune modulation

Probiotics are reported to enhance phagocytic activity of granulocytes and cytokine excretion in lymphocytes, increase immunoglobulin-secreting cells, and attenuate inflammasome activation. They are able to affect cells involved in immune responses, including epithelial cells, dendritic cells (DCs), T cells, regulatory T (Treg) cells, monocytes/macrophages, immunoglobulin A (IgA)-producing B cells, and natural killer cells [62, 63].

Probiotic bacteria have an effect on intestinal DCs, which have the ability to recognize and respond to different bacteria by linking the innate immune system to the adaptive immune response and to develop T- and B-cell responses. Badia et al. found that the immunomodulatory role of *S. boulardii* in the DCs prior to infection was related to the upregulation of tumor necrosis factor alpha (TNFα) and C—C chemokine receptor type 7 mRNAs, which might make the DCs more effective in antagonizing bacteria [64, 65]. Smith et al. reported that *S. boulardii* stimulated the production of cytokines TNFα, IL-1, IL-12, IL-6, and IL-10 in DCs and also

induced high levels of costimulatory molecules CD80 and CD86, thus modulated the immune system and led to an efficient clearing of enteropathogenic bacteria from the blood stream coupled with a faster cytokine response [65, 66].

Probiotics also influence intestinal epithelial cells through interaction with Toll-like receptors (TLRs) and downregulate the expression of NF-κB and proinflammatory cytokines [67, 68]. This effect is supported by the following studies: the supernatant of probiotic *Faecalibacterium prausnitzii* inhibited the NF-κB pathway *in vitro* and *in vivo* and showed protective effects in different models such as dinitrobenzene sulfate (DNBS)-induced colitis model and dextran sodium sulfate (DSS)-induced colitis [69]; the probiotic strain *L. rhamnosus* GG prevented cytokine-induced apoptosis in intestinal epithelial cells [70]; and *L. rhamnosus* GR-1 reduced the adhesion of *E. coli* by promoting TLR2 and NOD1 synergism and attenuating ASC-independent NLRP3 inflammasome activation [71].

3. Probiotic as antifungals for prevention and treatment of candidiasis

Candida is an opportunistic pathogen, causing mucosal infections including infections in the oral cavity, oropharynx, esophagus, and vagina, and potentially life-threatening systemic candidiasis. *Candida albicans* is the most common fungal pathogen in humans responsible for causing superficial as well as deep invasive candidiasis, which are essentially caused by *Candida* biofilms attached to body surfaces. Other *Candida* species such as *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida glabrata* are less frequently isolated in healthy and diseased humans [72–74]. Probiotics are known to reduce *Candida* infection in different organs and are generally considered to be beneficial for overall health. They appear to assist the host combat the pathogen by suppressing filamentation formation and reducing biofilm development, the mechanism of which may be related to expression of genes associated with biofilm formation and filamentation in *Candida* species. *In vitro* and *in vivo* studies have demonstrated the role of probiotics in the prevention of *Candida* colonization and invasive candidiasis [38, 75–86].

3.1. *In vitro* evidences: probiotics in prevention/treatment of *Candida* infections

Several *in vitro* studies have addressed the antifungal effects of probiotics against *Candida* isolated from the human oral cavity, GI tract, and genitourinary tract [77–81, 86, 87]. The probiotics that have been investigated against *Candida* species include *Lactobacillus* (e.g., *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *L. acidophilus*, *L. paracasei*, *L. johnsonii*, and *L. salivarius*), *Bifidobacterium* (e.g., *B. bifidum* and *B. infantis*), *Saccharomyces* (e.g., *S. boulardii*), and *Streptococcus* (e.g., *S. thermophilus*). **Table 3** shows candidacidal activity of probiotic strains in different studies. *C. albicans* appears to be more susceptible to the antifungal effect of *Lactobacillus* than *C. pseudotropicalis* [81], and the probiotics exhibited growth inhibitory activities against *C. glabrata, C. krusei*, and *C. parapsilosis* [79, 87].

Probiotics	Target pathogen	Study outcome	References
14 strains: *L. fermentum*, *L. rhamnosus*, *L. plantarum*, and *L. acidophilus*	*C. albicans* and *C. pseudotropicalis*	All probiotics inhibited the growth of *C. albicans* by H_2O_2 production and alternative mechanism	[81]
S. boulardii	*C. albicans* SC5314	*S. boulardii* inhibited the affecting hyphae formation, *Candida* adhesion, and biofilm formation by capric acid production	[87]
L. paracasei IMC 502	*C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*	High activity toward *Candida* strains except *C. glabrata* and *C. tropicalis*	[79]
L. plantarum ATCC 8014 and *L. johnsonii* enriched or not with SeNPs	*C. albicans* ATCC 14053	Strong inhibition of *C. albicans* by supernatant of selenium-enriched *Lactobacillus* spp.	[86]
L. acidophilus, *L. rhamnosus*, *L. salivarius*, *B. bifidum*, *S. thermophiles*, and *B. infantis*	*C. albicans* 10341	Significant inhibitory effect on biofilm formation and reduce viability of *Candida*	[80]
L. rhamnosus GR-1 *and L. reuteri* RC-14	*C. albicans* SC5314	Visible inhibition zones of fungal *C. albicans* by probiotic treatment; low pH environment caused by lactic acid and the H_2O_2 production may be anti-*Candida* factors	[77]
L. acidophilus ATCC 4356	*C. albicans* ATCC 18804	Reduce growth of *C. albicans* cells by 45.1%	[78]
L. casei subsp. *rhamnosus*	*Candida* spp.	80 preterm neonates with a very low birth weight: probiotic reduced incidence and intensity of enteric colonization by *Candida* spp. (RCT)	[28]
L. rhamnosus GG, *L. rhamnosus LC705*, *P. freudenreichii* subsp. *shermanii* JS	*Candida* spp.	276 elderly people: probiotic intervention reduced the risk of high yeast counts by 75% and the prevalence of hyposalivation (RCT)	[76]
L. rhamnosus GR-1 *and L. reuteri* RC-14	*Candida* spp.	55 women: probiotics significant reduced vaginal discharge, itching, and/or burning vaginal feeling, dyspareunia, and/or dysuria, and reduced the presence of *Candida* spp. (RCT)	[82]
L. acidophilus, *L. rhamnosus*, *B. longum*, *B. bifidum*, *S. boulardii*, *and S. thermophilus*	*Candida* spp.	150 children (aged 3 month to 12 year) on broad-spectrum antibiotics for at least 48 h: probiotic therapy avoided a significant increase in the number of patients colonized by *Candida* spp., significantly reduced the presence of *Candida* in the urine (RCT)	[83]
L. bulgaricus, *B. longum*, *and S. thermophilus*	*Candida* spp.	65 patients with *Candida*-associated stomatitis: detection rate of *Candida* spp. was reduced in the probiotic group; significant relief of clinical signs and symptoms after probiotic administration (RCT)	[84]

Probiotics	Target pathogen	Study outcome	References
L. acidophilus, B. lactis, B. longum, and B. bifidum	*Candida* spp.	112 preterm neonates (gestational age < 37 wk and birth weight < 2500 g): probiotics may reduce enteral fungal colonization and invasive fungal sepsis in low-birth-weight neonates (RCT)	[75]
L. reuteri DSM 17938 and *L. reuteri* ATCC PTA 5289	*Candida* spp.	215 elderly people (aged 60–102 y): significant reduction of *Candida* cells in saliva and plaque (RCT)	[85]

Table 3. Probiotics in prevention/treatment of ***Candida*** infections.

However, the mechanisms involved in antifungal activity of probiotics against *Candida* remain unclarified. Strus et al. found that *Lactobacillus* strains could inhibit the growth of *C. albicans* to a certain degree and their anticandidal activity related to H_2O_2 production [81]. Murzyn et al. reported that *S. boulardii* was able to secrete active compounds, mainly capric acid, reduced the expression of *hwp1*, *ino1*, and *csh1* genes that encode virulence factors in *C. albicans* SC5314 cells, and inhibited filamentation of *C. albicans* and its mycelial development [87]. Therefore, it is likely that the antimicrobial molecules, organic acids, and H_2O_2 produced by probiotic are major factors to limit growth of fungal pathogen *Candida.* This idea was supported by the research of Köhler et al. They demonstrated that low pH environment caused by lactic acid and the H_2O_2 production of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 strains played important role in their inhibition activity to *C. albicans* SC5314. Moreover, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 inhibited genes associated with *C. albicans* biofilm formation [87]. This result, together with the findings in Murzyn et al. study, shed light on a novel approach for uncovering the molecular mechanisms of the probiotic effect by using gene expression and related technology.

3.2. *In vivo* evidences: probiotics in prevention/treatment of *Candida* infections

In vivo studies, especially RCTs, have also been performed to substantiate the antifungal activity of probiotics in humans. These studies mostly focus on the sites of oral cavity, GI tract, and urogenital tract, which are susceptible to *Candida* infections (**Table 3**).

The elderly are a group particularly susceptible to oral candidiasis, because of frequent usage of dentures, hyposalivation, and their weakened immune status. Researches by Hatakka et al. and Kraft-Bodi et al. have shown that the daily consumption of food with *L. reuteri* DSM17938, *L. reuteri* ATCC PTA 5289, and *L. rhamnosus* GG ATCC 53103 significantly reduced the high yeast counts in saliva and biofilms in the elderly [76, 85]. The removal of biofilms by the use of probiotics that reduce the oral burden of *Candida* could play a major role in preventing oral candidiasis in denture wearers.

For the urogenital tract, chronic vulvovaginal candidiasis (VVC) is the most common candidiasis disease and impacts the life quality of thousands of women around the world. Researches on the effect of probiotics in the treatment and prophylaxis of VVC have been performed [82]. Martinez et al., in an RCT involving 55 women, demonstrated that the administration of *L. rhamnosus*

GR-1 and *L. reuteri* RC-14 significantly reduced the presence of *Candida* and therefore reduced the vaginal discharge, itching, and/or burning vaginal feeling, dyspareunia, and/or dysuria [82].

For the GI tract, *Candida* species are common inhabitants of GI tract. Dysbiosis of GI tract may lead to candidal overgrowth and possible invasive infections, especially in infants. Hence, immunocompromised children, especially preterm neonates with low birth weight, have been the target population of a large number of studies to evaluate the prevention or/and treatment potentials of probiotics to *Candida* infections [28, 75, 83]. Manzoni et al., in an RCT involving 80 very low birth weight (VLBW) neonates, demonstrated that orally administered *L. casei* subsp. rhamnosus significantly reduced incidence and intensity of enteric colonization by *Candida* [28]. Another RCT, by Roy et al., found *L. acidophilus*, *B. lactis*, *B. longum*, and *B. bifidum* reduced enteral fungal colonization and invasive fungal sepsis in 112 preterm neonates (gestational age < 37 wk and birth weight < 2500 g) [75].

Together, both the laboratory studies and clinical studies showed that probiotics could prevent *Candida* colonization by inhibiting adhesion, filamentation, and biofilm formation, and therefore supplementation of probiotics could be a potential approach for reducing *Candida* colonization and invasive candidiasis.

4. Safety of probiotics

Although most commercially available probiotic strains are generally regarded as safe and none of the clinical studies mentioned above were reported to have adverse effects directly related to probiotics, there are some concerns regarding the safety of probiotics, including potential of bacteremia and/or endocarditis occurrence, toxicity to the gastrointestinal tract, and transfer of antibiotic resistance [4].

4.1. Potential of bacteremia and/or endocarditis occurrence

Lactic acid bacteria, including *Bifidobacterium*, have been reported to cause bacteremia as well as endocarditis [88–92]. Cannon et al. described that *L. rhamnosus* caused liver abscess, lactobacillemia, and infective endocarditis in a few case studies, and also the occurrence of *Lactobacillus* sepsis was directly linked with the ingestion of probiotic supplements, especially among immunocompromised patients and those with endocarditis [89]. Kunz et al. found two premature infants with short gut syndrome developed Lactobacillus bacteremia while taking *Lactobacillus* GG supplements. However, the risk of infection due to Lactobacilli is extremely rare. Statistic data from surveillance in Finland suggest that there was no increase in *Lactobacillus* bacteremia during 1990–2000, and Lactobacilli were isolated in 0.02% of all blood cultures [93].

4.2. Toxicity to the gastrointestinal tract

The role of probiotics on gastrointestinal physiology suggests a theoretical possibility that the production of metabolites might be undesirable and also might lead to malabsorption due to deconjugation of bile salts. These might increase the risk of colon cancer; however, there is no epidemiologic or clinical evidence to support this hypothesis [94, 95].

4.3. Transfer of antibiotic resistance

Another major safety concern of theoretical importance is genetic transfer of antibiotic resistance from probiotic strains to pathogenic cells in the gastrointestinal tract [96, 97]. Plasmids with antibiotic-resistance genes, including genes encoding resistance to tetracycline, erythromycin, chloramphenicol, and macrolide-lincosamide-streptogramin, have been found in *L. plantarum*, *L. fermentum*, *L. acidophilus*, and *L. reuteri* strains. *L. plantarum* 5057 exhibited tetracycline resistance, and *L. lactis* was with streptomycin, tetracycline, and chloramphenicol resistances [98–100]. Although the transfer of native *Lactobacillus* plasmids is quite rare, there are some cases, e.g., the antibiotic-resistance plasmids from *Lactococcus* species could transfer to *Leuconostoc* species and *Pediococcus* species.

With respect to the potential risks of probiotics, it is important to conduct population-based surveillance for safety concern.

5. Conclusions

Probiotics have the ability to restore the imbalance of intestinal microbiota and could act as both prophylactic and adjunctive therapy against candidiasis. Antifungal effect of probiotics is likely due to their interference with *Candida* biofilm development and hyphal differentiation. Safety may be of concern in application, as probiotic strains may, although quite rarely, cause bacteremia, fungemia, and sepsis. Well-designed RCTs are required to address these issues before the routine use of probiotics is recommended.

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Chapter 2

A Network of Physiological Interactions Modulating GI Homeostasis: Probiotics, Inflammasome, mTOR

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Additional information is available at the end of the chapter

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Abstract

The gastrointestinal surface is in constant interaction with various exogenous molecules. Exogenous components are discriminated in the GI context, as good, in case of nutrients and fibers, and bad, when they negatively affect host integrity. During this tolerogenic process, they also train the host's immune system. The immune system is a morpho-physiologic unit driven by immune cells with the assistance of commensal organisms. Several species of commensal microorganisms have been used for centuries as probiotics due to their beneficial effects on human health. Lowering local levels of pro-inflammatory cytokines has a systemic effect, which is one of the fundamental characteristics associated with probiotics. Still, the primary mechanisms wiring those regulatory circuits as a unit remain unclear. Modulation of the innate immune system, via regulation of inflammasome assembly is emerging as a critical driver of this interaction. Stimulation of toll like receptors (TLR) and inner cell sensors like NLRP3 connect probiotics with essential host systems. In this context, the mTOR-regulated circuits, an intricate network modulating a cascade of protein phosphorylations, could be an important channel connecting host metabolism and probiotics crosstalk.

Keywords: *Lactobacillus*, inflammasome, caspase-1, mechanistic target of rapamycin (mTOR), insulin resistance, adipogenesis, type 2 diabetes, cancer

1. Overview of probiotics

1.1. History and use

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, as defined by the World Health Organization [1]. This is an extremely

broad definition that encompasses fungal and other eukaryotic species, as well as bacteria. In practice, however, bacterial probiotics receive the most attention. Bacterial probiotics can be found as various supplements and food additives in products such as pills and yogurts [2]. The benefits of probiotic supplements have been recognized for centuries, long before it was understood that the living microorganisms in the supplement provided the benefit. Fermented milk products were used as a treatment for intestinal discomfort in the Roman empire, and ancient Chinese scholars recommended fecal transplant to combat diarrhea [3]. Today, probiotics are often prescribed by gastroenterologists and GI surgeons to help alleviate irritable bowel syndrome, pouchitis, and functional diarrhea, however the potential applications of probiotics in other systems is gaining notice [4]. Yogurt and other fermented milk products as well as probiotic drink mixes are commonly used forms of probiotic supplements today [4].

Strains of the genera *Bifidobacterium* and *Lactobacillus* are the most common bacteria studied and used as probiotics, however *Enterococcus, Streptococcus, Leuconostoc, Bacillus*, and even the yeast *Saccharomyces boulardii* have been used [5, 6]. Knowledge of both the species and strain of bacteria is important in the study and use of probiotics as different strains can produce varying effects on the host. For instance, *Escherichia coli* Nessile 1917 is a beneficial probiotic while *E. coli* 0157:H7 is a deadly pathogen [2, 5]. Sources of probiotics vary. Probiotic bacteria are commonly found in fermented milk products, which lactic acid producing bacteria are essential to the production of, and they have also been isolated from stool samples of healthy individuals [5].

1.2. Mechanisms of action

Probiotics can have a wide array of beneficial effects on their host organism (**Figure 1**). One way in which probiotics can benefit the host is to simply prevent or reduce the probability of infection by pathogenic organisms. By forming aggregates with intestinal pathogens, probiotics can reduce the ability of these pathogens to adhere to the intestinal mucosa and initiate infection [7]. *Saccharomyces boulardii*, *Lactobacillus gasseri* 4B2, and *Lactobacillus coryniformis* DSM 20001^T have shown the ability to aggregate with pathogenic strains of *E. coli* (serogroup 0157:H7, and serogroup K88, respectively) [7, 8]. Probiotic bacteria can also increase mucin production in the gut, further reducing ability of pathogens to adhere to and infect host epithelial cells [9]. *E. coli* Nessile 1917 can upregulate the production of MUC2 and MUC3, the primary mucins present in the human colon [10]. Probiotic bacteria often have the ability to produce molecules damaging to pathogens, protecting the host organism by killing or inhibiting the activity of pathogenic bacteria. Several *Lactobacillus* strains produce antimicrobial bacteriocins, some examples include acidocin produced by *Lactobacillus acidophilus*, and sakacin produced by *Lactobacillus sakei* [11, 12]. These molecules may help the host maintain gut homeostasis by regulating the gut bacterial community. Several *Lactobacillus* species can inhibit the growth of *Clostridium difficile* or *C. perfringens* through the production of organic acids, and *Lactobacillus plantarum* LPAL and *Bifidobacterium animalis* ssp. *lactis* BLC1 produce some unknown bactericidal compounds or bacteriocins that inhibit both species [13]. Beneficial gut bacteria can also induce host immune cells to produce defenses against pathogens. Gut bacteria stimulate the production of an antibacterial, peptidoglycan-binding lectin in mice and in humans [14].

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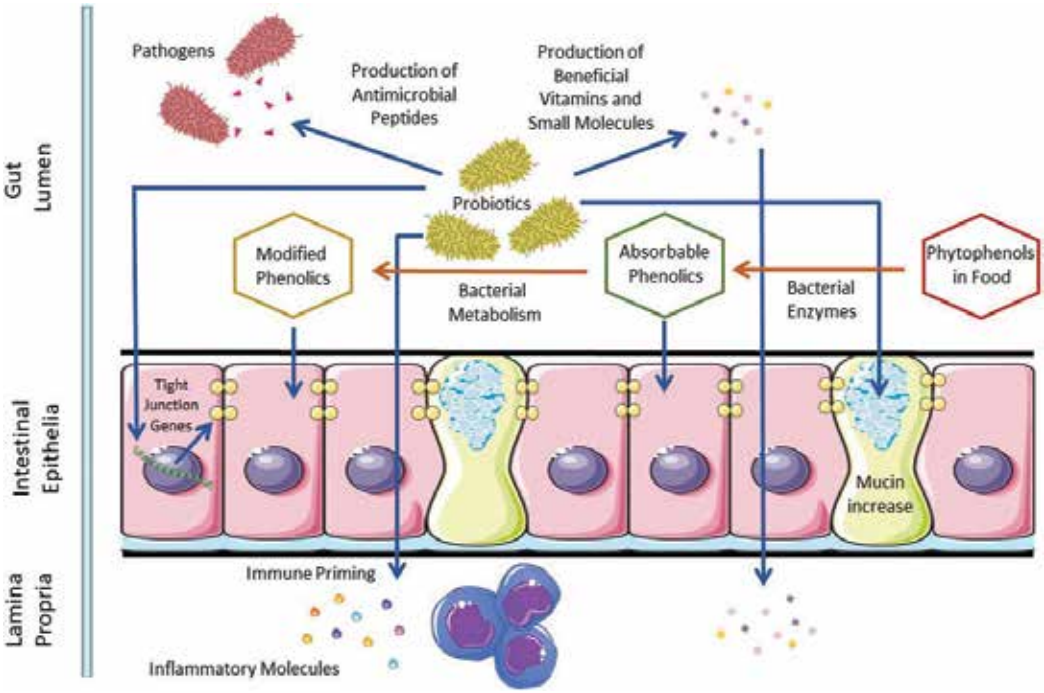

Figure 1. Schematic of possible mechanisms of probiotic interactions with molecules in the intestinal lumen as well as host epithelial cells.

Probiotics can also inhibit the growth of pathogens in other ways. *Lactobacillus delbrueckii* can bind iron to its surface, making it unavailable to pathogens, many of which need iron to survive [6]. Probiotics may also benefit the host by reducing the ability of pathogens to diffuse across epithelial cell barriers: strains of *Lactobacillus* show an ability to increase intestinal barrier function. Recent research has documented an increase in the levels of claudin-1 and goblet cells seen in healthy rats as well as in *Lactobacillus johnsonii* fed animals, suggesting that one aspect of the bacteria's role in the gut is to strengthen the barrier function to prevent a leaky gut and maintain a high level of mucin production to protect the gut epithelial cells [15]. *Lactobacillus johnsonii* also appeared to increase the expression of inflammatory chemokines, including CCL20 (MIP3A), CXCL8 (IL-8), and CXCL10 (IP10) [16]. This result may indicate that exposure to beneficial *Lactobacillus* primes the gut immune system so that it is resistant to overwhelming inflammation in the face of later insults [16]. An increase in Paneth cells, immune cells in intestinal crypts, was also demonstrated in *Lactobacillus* fed animals [16]. Overall, probiotic bacteria, many in the genus *Lactobacillus*, can play an important role in defending the gastrointestinal tract from pathogenic organisms.

Probiotics can exert their positive effects on the host by producing vitamins or other materials useful to the host: *Bifidobacterium adolescentis* and *B. pseudocatenulatum* produce B vitamins including B1, B2, B3, B6, B8, B9, and B12 [6]. Probiotics may also increase the availability of nutrients already present in foods. Lactic acid bacteria increase the amount of available folic acid in fermented milk products [9]. The positive effects of Lactobacilli may also result from

the bacterial production of esterases. These enzymes are produced by Lactobacilli and have the ability to release beneficial phenolic compounds, such as ferulic acid and caffeic acid, from food molecules [17]. *Lactobacillus johnsonii* N6.2, a strain associated with diabetes resistance in BioBreeding diabetes prone and diabetes resistant rats, produces two ferulic acid esterases that cleave ethyl ferulate and chlorogenic acid [17]. Other small molecules increased by probiotic bacteria can include free amino acids, and short chain fatty acids such as lactic acid, propionic acid, and butyric acid, which can be used by host cells for energy [9]. Some strains of *Lactobacillus* can produce hydrogen peroxide, which is beneficial to the gastrointestinal tract when present in small amounts [18]. In the case of host lactose intolerance, some strains of lactic acid bacteria, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* can aid in the host's digestion of lactose by supplementing host lactase with their own [9]. *Lactobacillus* species can also increase the nutritional value of various food products. Fermentation with several *Lactobacillus* strains increased the dietary phenol available in cereal grains by a considerable amount [19]. Through both the synthesis and the breakdown of various substances, probiotics can improve host nutrition.

Probiotics have also shown promise in the area of cancer research. *Lactobacillus casei* and *L. rhamnosus* GG can reduce invasion in colon cancer cells, a key property in preventing metastasis [20]. Levels of matrix metalloproteinases, implicated in cell invasion, can be responsive to probiotic treatment: *Lactobacillus acidophilus* and *L. rhamnosus* GG can decrease the expression of matrix metalloproteinase-9 by increasing the expression of the tissue inhibitor of metalloproteinases [20]. Treatment with kefir reduces the viability of colon cancer cell lines by inducing apoptosis and the proliferation of colon cancer cell lines by arresting the cell cycle in the G1 phase [21]. These results suggest that probiotics may be useful in the treatment or prevention of some cancers.

1.3. Health benefits

Probiotics are commonly used for gastrointestinal complaints and issues, and there has been extensive research on the benefits of probiotics in this body system. Modern research often supports the old assertions that consumption of probiotics is beneficial to gastrointestinal health. Probiotic supplements have shown efficacy in treating certain intestinal disorders in animal models and in humans. Patients in remission from pouchitis who received probiotic treatment in the form of a bacterial supplement called VSL#3 showed increased *Bifidobacterium* and *Lactobacillus* diversity compared to patients receiving a placebo treatment [22]. *Bifidobacterium* and *Lactobacillus* are commonly regarded as beneficial members of the gut microbiota [23]. VSL#3 was also found to reduce the frequency of pouchitis recurrence [24]. This provides support for the use of probiotics in the treatment of GI diseases. A fermented soy probiotic mixture was shown to provide multiple gastrointestinal health benefits to rats with induced colitis. Rats fed the probiotic mixture of *Bifidobacterium longum* and *Lactobacillus helveticus* 416 had no colon damage, ulcers, or swelling, compared to rats who did not receive the probiotic supplement [25]. The rats receiving the probiotic also showed increased intestinal *Lactobacillus* and *Bifidobacterium* populations [25]. Supplementation with probiotics can help adjust the gut microbiota, and this likely plays a role in the effects of diseases of the

gut. Apple juice fermented with *Lactobacillus* species showed the ability to inhibit *Helicobacter pylori in vitro*, but did not negatively affect other positive GI bacteria [26]. This further shows the ability some probiotics have to ameliorate disease-induced tissue damage and regulate the gut microbiota.

Probiotic supplements are no cure-all for gastrointestinal maladies, however. Assorted studies have reported little to no benefit of probiotics in the treatment of other gastrointestinal diseases. *Lactobacillus* probiotics were not shown to be an effective treatment in helping patients with Crohn's disease stay in remission [27]. In a clinical trial involving women with irritable bowel syndrome, treatment with probiotics was not more effective than the administration of a placebo in reducing IBS symptom severity [28].

On the other hand, there is also a wealth of research showing probiotics to have benefits in areas of the body besides the adult gut. The importance of an individual's microbiome is evident even before birth, therefore the prenatal and neonatal use of probiotics is an important consideration in infant health. The systemic benefits of probiotics can be transferred from mother to infant. In a study on allergies, a probiotic combination taken by an allergic mother, consisting of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12, decreased the probability of sensitization in breastfed infants, possibly by increasing the concentration of the anti-inflammatory cytokine transforming growth factor-beta 2 (TGF-β2) in breast milk [29]. Here we see the ability of a probiotic to induce immune changes in one organism that can be transmitted and positively affect the health of another.

Certain strains of bacteria have also been shown to reduce the negative effects of oral infections. In a study involving mice that were intubated with *Lactobacillus gasseri* SBT2055 and then infected orally with *Porphyromonas gingivalis*, the intubated mice showed less alveolar bone loss and better maintenance of the periodontal ligament than non-intubated mice [30]. In this case, pretreatment with probiotics helped prevent oral damage from infection. Probiotics may also help maintain or improve liver health. A probiotic mixture containing *Bifidobacterium* and *Lactobacillus* species reduced weight gain, maintained intestinal barrier function, and reduced liver inflammation in rats fed an inflammation-inducing high fat diet [31]. Another study using various *Bifidobacterium* strains corroborated these findings. *B. pseudocatenulatum* LI09 and *B. catenulatum* LI10 showed the ability to reduce D-GalN-induced liver damage and serum levels of inflammatory cytokines in rats [32]. Fang et al. found that supplementation with probiotics reduced levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), improved liver necrosis and inflammatory cell infiltration, reduced bacterial translocation to mesenteric lymph nodes, and reduced levels of interleukin 1β, macrophage inflammatory protein 1α, monocyte chemoattractant protein 1, and macrophage colony-stimulating factor in rats [32].

Probiotics have been used for centuries around the globe to improve health and treat disease. Although they are most commonly used to treat gastrointestinal diseases, they can exert positive effects on the health of the entire host organism. Although there has been much research elucidating how probiotics benefit their host and what benefits they actually provide, there is still much to be discovered about the many potential benefits of probiotics.

2. The effects of probiotics on the inflammasome

2.1. Inflammasome: the interface between detection and response in inflammation

Inflammation is a complex immune response to many different insults, such as pathogens, cell death, and chemicals, which promotes survival during infectious diseases or injuries, as well as maintains tissue homeostasis. When an insult is identified, a cascade of signals is triggered, concluding in the recruitment of neutrophils and macrophages, which have the ability to produce several cytokines and chemokines. Despite the beneficial effects of inflammation, it must be tightly regulated, otherwise it may lead to serious tissue damage due the overproduction of inflammatory cytokines [33]. The secretion of cytokines is regulated at the transcriptional level, and many of them are also regulated at the posttranslational level [34]. Considering that the exposure to pathogens and chemicals is the first step in inflammation, the gastrointestinal environment has a crucial role in this process. Gut epithelial cells are the first cells to be exposed to both microbiota and food components, leading these cells to be key players influenced by food antigens, pathogens, toxins, and also by bodily metabolism and functions. Furthermore, the gut epithelial cells are the first line of defense against pathogens, complementing the action of the associated mucosal immune system, the development and maintenance of which are induced by the microbiota [35]. Some intestinal diseases are largely affected by the gut microbiota, such as inflammatory bowel disease (IBD), and Crohn's disease (CD) [36].

2.2. Components of inflammasomes

The mechanisms to identify an insult and trigger an immune response may vary according the kind of the antigenic molecule. In order to identify different antigen molecules, the innate immune cells of mammals can detect these molecules through a fixed number of germline-encoded pattern recognition receptors (PRRs), which have the ability to recognize microbial structures called pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acid and bacterial cell wall [37]. Furthermore, damaged host cells can release some molecules termed danger-associated molecular patterns (DAMPs), such as ATP, reactive oxygen species (ROS) and uric acid, which also have the ability to trigger PRRs [38]. Some PRRs are located in the cell membrane and endosomes and are called toll like receptors (TLRs) and C-type lectin receptors (CLRs), which are able to recognize PAMPs and DAMPs located in the extracellular milieu. The other class of receptors is the NOD-like receptors (NLRs), which are located inside the cell in the cytoplasm [39].

TLRs were first characterized by Christiane Nusslein-Volhard in 1985, when she observed that the protein encoded by *Toll* gene was responsible for preventing the dorsoventral patterning in *Drosophila* embryos [40]. Later, it was observed that TLRs trigger a specific response for different microbes, ending up in the activation of specific regulatory pathways [41]. To date, several TLRs have been classified in mammals and theirs targets identified. Highly conserved, TLRs belong to type 1 transmembrane glycoproteins and are composed of three main structural components: a leucine-rich motif for ligand recognition at N-terminus; a single transmembrane helix; and a cytoplasmic Toll/interleukin-1 (IL-1) receptor domain at C-terminus, as reviewed by Gao and coworkers [42]. TLRs can be expressed on cell membrane, as well as on endosomal membrane. The

TLRs expressed on cell membrane are TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11 and TLR12, whereas TLR3, TLR7, TLR8, TLR9 and TLR13 are expressed on endosomal membrane [43]. Each TLR specifically binds to microbial molecules, triggering a cascade of signals that result in the transcription and production of pro-inflammatory cytokines and chemokines. TLR4 is one of the most studied TLRs due its ability to detect lipopolysaccharide (LPS), leading to the activation of both myeloid differentiation antigen 88 (MyD88)-dependent and MyD88-independent pathways [44]. Downstream, MyD88 is responsible for the activation of the master transcriptional regulators MAPK and NF-κB, which increase transcriptional expression of IL-1β, IL-6, IL-8 and IL-18 [45].

Like TLRs, NLRs can sense different molecules and trigger an inflammatory response. NLRs also have a structure composed of three main domains: caspase recruitment domain (CARD) or pyrin domain (PYR) at N-terminal; the highly conserved NATCH domain, a nucleotide-binding domain (also called as NBD); and leucine-rich repeats (LRR) at the C-terminal [46]. Based on the N-terminal domain, NLRs are subdivided into 8 sub-families (**Figure 2**). The LRRs are responsible for microbial molecule detection, whereas the CARD and PYD domains are responsible for homotypic and heterotypic interactions of NLRs with downstream molecules, such as procaspase, directly or via the adaptor molecule, apoptotic-associated speck like protein (ASC) [47].

2.3. Inflammasome assembly

Once epithelial cells recognize PAMPs or DAMPs, many different responses can be triggered in order to eliminate the source of those molecules. One well-known response against pathogens is called the inflammasome. Inflammasomes are a multiprotein complex formed in response to PAMPs and DAMPs, resulting in the activation of caspase-1 (canonical pathways) or caspase-11 (non-canonical pathway) [48]. NLRs located in the cytosol act as sensors of these microbial molecules, leading to the activation of the inflammasome complex. The inflammasome is basically composed by the NLR family members, which may contain the PYR domain or just the CARD, and by the adapter ASC. ASC has both CARD and PYD domains, and the association between ASC and CARD-containing NLRs recruits caspase-1 via homotypic interactions [49]. Despite around 23 NLR genes having been identified to date, only some of them can form oligomeric complexes which end up in the post-translational activation of caspases [50]. The hallmark of the inflammasome is the recruitment of caspase-1 in the canonical pathway, which is further released and subsequently activated via auto cleavage. Active caspase-1 can cleave and activate more than 70 substrates. This sequential process will finally release active caspase-1 to activate the IL-1β cytokine and gasdermin-D to promote adaptive and humoral immunity (**Figure 2**) [51]. In the non-canonical pathway, cleavage and activation of interleukins can also occur via caspase-4/caspase-5 (humans) or caspase-11 (rodents) [52].

Despite the fact that caspase-1 is a protein that plays an important role in many different pathways, one of the most studied ones is pyroptosis, which is a cell death caused by inflammation in response to microbial infections or nonmicrobial stimuli [53]. In pyroptosis, caspase-1 is activated through inflammasome assembly and its active form can then cleave gasdermin-D (GSDMD) at Asp276, which generates the N-terminal cleavage product (GSDMD-NT) triggering pyroptosis and cell death. GSDMD-NT has the ability to form pores on the cell membrane, leading to cell leakage and the release of pro-inflammatory cytokines [54]. Moreover, active

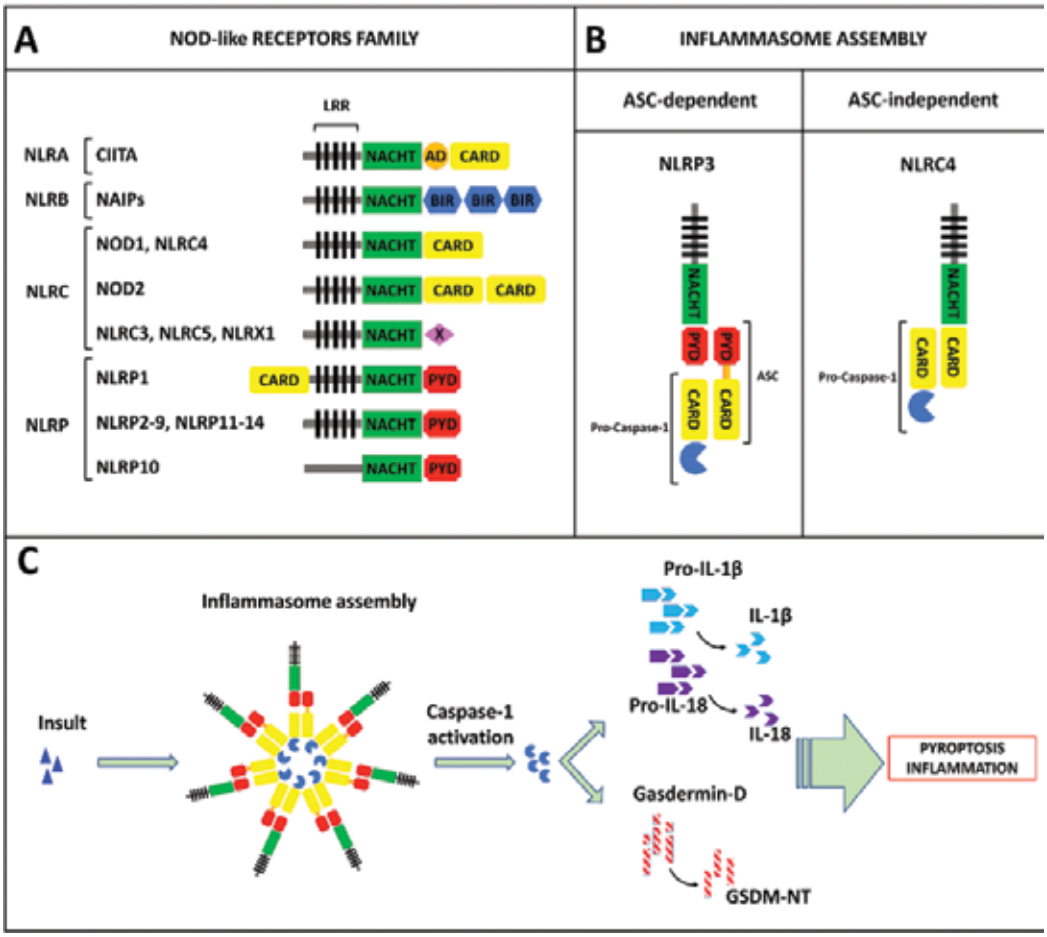

Figure 2. NLRs families and triggering of the inflammasome. (A) NRRs currently known families, showing the highly conserved NACHT domain; (B) differences between ASC-dependent and ASC-independent binding to caspase-1; (C) schematic activation of the inflammasome.

caspase-1 can also cleave pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their active form. IL-1β is a pyrogenic cytokine that can promote adaptive and humoral immunity. Neither IL-1β nor IL-18 are secreted by the endoplasmic reticulum-Golgi route. Nevertheless, IL-18 is constitutively expressed in macrophages, whereas IL-1β expression is regulated by NF-κB-mediated transcription [48]. There are other signals that can also trigger the auto-cleavage of pro-caspase-1 independent of NLRP3 activation. Some examples of these secondary signals are ROS and unfolded proteins [55].

2.4. Dysbiosis and inflammasomes

The gastrointestinal system harbors a diverse and complex microbial community that has a pivotal role in host health. However, changes in the microbiota population can have major consequences, beneficial or harmful, for host health. The disruption of the gut microbiota, called

dysbiosis, has been observed in several pathological conditions such as obesity, diabetes, and IBD, encompassing ulcerative colitis (UC) and CD [56, 57]. In humans, susceptibility to type 1 diabetes has been associated with changes in the gut microbiota composition, with a significant augmentation of bacteria of the Bacteroidetes phylum, and lower concentrations of *Bifidobacterium, Lactobacillus*, and *Clostridium* strains [58]. The search for probiotic strains that can reestablish host health has strongly increased in the past decades. Most of the microflora of healthy hosts is composed of bacteria from four bacterial phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria [59]. The genus *Lactobacillus* belongs to the Firmicutes phylum, which explains the large amount of studies with *Lactobacillus* species being administered as probiotics.

The inflammasome has been considered as an important regulator of intestinal homeostasis, due the central role of IL-1β and IL-18 in Th1 responses by the induction of IFNγ [60]. Moreover, IL-1β is responsible for induction of neutrophil influx, activation of myeloid cells and lymphocytes, and stimulation of Th17 differentiation [61]. Despite the fact that activation of inflammasomes increases the maturation of the pro-inflammatory cytokines IL-1β and IL-18, there is some evidence that the inflammasomes are important for keeping intestinal homeostasis and reducing morbidity and mortality in dextran sulfate sodium (DSS)-induced colitis in mice. It has been shown that mice deficient in some NLRs, such as NLRP1, NLRP3, NLRP6, NLRP12, AIM2, or deficient in ASC exhibited higher levels of pro-inflammatory mediators, as well as an increase in the epithelial damage within the colon, as reviewed by Chen [60]. Surprisingly, the severity of DSS-induced colitis seems to be reduced when antibiotic therapy is provided to mice, which strongly suggests the role of the gut microbiota in the phenotype of inflammasome-deficient mice [62]. This result was also observed in another experiment where inflammasome-deficient mice were cohoused with wild-type mice or with mothers of the opposite phenotype. After some days living together, an increase in colitis transmissibility through microbial transfer was observed.

The mechanism by which inflammasomes can affect the gut microbiota composition is still unclear. However, the effects of IL-18 on the production of antimicrobial peptides (AMPs) have revealed a possible explanation. IL-18 is able to upregulate the production of AMPs, which is crucial for microbial clearance [63]. *Asc*$^{-/-}$, *caspase-1*$^{-/-}$, *AIM2*$^{-/-}$, *or Nllrp6*$^{-/-}$ mice have shown lower levels of AMPs when compared with WT, but normal levels of specific AMPs are restored after the administration of recombinant IL-18 [60]. Considering that AMPs can be produced to target a specific microbe, the modulation of AMP production can contribute to the abundance of certain bacterial populations. Administration of Ang4, a well characterized AMP, into *Asc*$^{-/-}$ mice changed the overall diversity and community of gut microbiome, but it was still significantly distinct from the WT mice [63]. All these data suggest that despite the activation of the inflammasome increasing the release of pro-inflammatory cytokines, shutting down this pathway also contributes to undesired inflammation. Thus, the modulation of the inflammasome seems to be a key factor in the prevention of exaggerated inflammation.

2.5. Probiotics and inflammasome

Many studies have focused on the use of probiotic strains that could avoid or ameliorate inflammation. One promising treatment for IBD is the commercially available probiotic mixture VLS#3,

which is a mixture of eight strains of lactic acid-producing bacteria (*Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *Bulgaricus, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis* and *Streptococcus salivarius* subsp. *Thermophilus*). VLS#3 has been shown the ability to ameliorate and prevent colitis in the $Il10^{-/-}$ murine model [64]. The mechanisms by which VLS#3 can reduce intestinal inflammation are still unclear, but several independent results have shown the effects of VLS#3 on the gastrointestinal tract. It was observed that the administration of VLS#3 decreases the biodiversity of the luminal microbiota on TNBS-induced chronic colitis rats [65]. Moreover, TNBS-induced colitis rats treated with VLS#3 have demonstrated pro-inflammatory cytokine and chemokine levels similar to the levels observed in normal rats [66]. These results are in agreement with the effects of VLS#3 observed on the inflammasome of NOD mice: decreasing the mRNA levels of *Il1b* and increasing the mRNA levels of *Ido*, an immunomodulatory enzyme, comparable to control group levels [67]. Surprisingly, VLS#3-treated NOD mice also reduced effective T cells/regulatory T cells (Teff/Treg) ratios at both systemic and pancreatic lymph nodes levels, helping in the maintenance of the immune homeostasis and avoiding excess inflammation.

Due to the proximity of the vaginal mucosa to the gastrointestinal system, the vaginal microbiota is largely affected by the gut microbiota, being dominated by Lactobacilli [68]. Recently, *Lactobacillus rhamnosus* GR-1 has been reported to be able to limit *Escherichia coli*-induced inflammatory response in Bovine Endometrial Epithelial Cells [69]. It was observed that *L. rhamnosus* reduces inflammation by downregulating *Tlr2, Tlr4, Nod1* gene expression, as well as the downregulation of *Myd88* and *Nfkb* mRNA levels. Moreover, this strain showed the ability to reduce mRNA levels of the main components of the inflammasome: NLRP3, ASC, and Caspase-1. Consequently, the mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-18, and TNFα where suppressed by *L. rhamnosus*.

The activation of the inflammasome seems to not be dependent on bacterial viability or require phagocytosis, but the potassium efflux seems to be crucial. A study with bone marrow-derived macrophages (BMDMs) incubated with heat-killed *B. infantis* did not show an increase in the IL-1β levels when compared to cytokine levels when BMDMs were incubated with live bacteria. However, when the cells were incubated with heat-killed bacteria overnight, the IL-1β levels were similar to the levels observed when incubated with live bacteria [70]. In the same work, it was observed that using cytochalasin D, a phagocytosis inhibitor, did not significantly change the IL-1β levels. Interestingly, when WT macrophages were incubated with high concentrations of potassium or with the potassium channel blocker ruthenium red, the levels of IL-1β were significantly lower in response to *B. infantis* or *B. fragilis*, suggesting that the activation of NLRP3 inflammasome is dependent on potassium efflux.

The modulation of the inflammasome by probiotics or gut microbiota does not only affect the gastrointestinal system. In fact, the gut microbiota can modulate the inflammasomes and its effects systemically. The concentrations of pro- and anti-inflammatory cytokines have been correlated with some neurological pathologies, such as depression, which is characterized by high levels of pro-inflammatory cytokines (i.e. IL-1β and IL-6) and low levels of anti-inflammatory cytokines (i.e. IL-4 and IL-10) [71]. Moreover, more IL-1 receptor type-I and its ligands have been found to be highly expressed in brain areas related to stress response, and chronic stress

and the administration of IL-1β have been characterized as triggers of depression-like behavior [72]. Nevertheless, higher levels of caspase-1 and NLRP3 mRNA have been observed in blood cells of depressed patients, which suggests that the inflammasome pathway may play a key role in the development of depression [73]. *Casp1*$^{-/-}$ mice showed decreased depressive and anxiety-like behaviors after a forced swim test compared with WT mice [74]. The effects of chronic restraint stress assay, which increases the caspase-1 and IL-1β levels, also resulted in altered gut microbiota compared to non-stressed mice. The relative abundances of the genera *Allobaculum, Bifidobacterium, Turicibacter, Clostridium,* and the family S24-7 were significantly reduced in restrained animals, whereas the abundance of the family Lachnospiraceae showed an increase. *Bifidobacterium* spp. is a genus associated with the suppression of inflammation by the inhibition of the nuclear factor-κ-B (NF-κB) pathway [75]. All these findings strongly support the notion that the inhibition of caspase-1 can reduce the stress response by modulating the interface between stress and the gut microbiota, and that the gut microbiota can exert some important effects on brain function via the inflammasome signaling.

In the past decade, many studies have demonstrated the effects of the gut microbiota on metabolic diseases. In comparing the gut microbiota of two distinct kinds of rats, the Biobreeding Diabetes Prone (BB-DP) and the Biobreeding Diabetes Resistant (BB-DR) rats, a higher abundance of *Lactobacillus* and *Bifidobacterium* species was identified in BB-DR stool samples [56]. One of the most prevalent species found in this work was *Lactobacillus johnsonii*, which was isolated from the stool of BB-DR rats. *L. johnsonii* has two cinnamoyl esterases that utilizes many phenolic compound as substrates [17]. One well known substrate is rosmarinic acid (RA), a phenolic compound extracted from diverse kinds of plants from the Nepetoideae subfamily of the Lamiaceae family [76]. These cinnamoyl esterases can cleave RA into its two components, caffeic acid (CA) and 3,4-dihydroxyphenylactic acid (DOPAC). Both RA and its components are well known for their antioxidant and anti-inflammatory properties [77, 78]. Based on the activity of the cinnamoyl esterases on RA, a recent study compared the effects of *L. johnsonii* N6.2 when administrated alone or in combination with RA on the inflammasome pathway in the ileum tissue of BB-DP rats fed daily with these treatments. It was observed that, despite higher levels of caspase-1 mRNA and higher levels of pro-caspase-1 in the rats fed with *L. johnsonii* N6.2, this strain decreased the concentration of the active caspase-1, compared to the animals fed with RA alone or in combination with the bacterium [79]. In the same study, it was observed that only RA significantly induced the expression of the *Il1b* gene, 12.5-fold compared to the PBS control. Consequently, RA-fed rats accumulate higher amounts of total IL-1β in the tissue. Lower levels of the pro-inflammatory cytokines TNFα and IFNγ were also observed in BB-DP rats fed with *L. johnsonii* N6.2 [80]. A similar result was observed in dogs with chronic enteropathy (CE) that were treated *ex-vivo* and *in-vivo* with *Enterococcus faecium* [81]. It was observed that *ex-vivo* stimulation of duodenal biopsies with *E. faecium* increased the mRNA levels of caspase-1 in CE dogs. However, the protein levels of IL-1β was significantly reduced after treatment. Moreover, *L. johnsonii* N6.2 demonstrated to be able to produce H_2O_2, which has an inhibitory effect on the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is the rate-limiting enzyme of tryptophan catabolism, converting tryptophan into *L*-kynurenine. The accumulation of cytotoxic kynurenines due to higher IDO activity can result in localized immunosuppression [82]. All these anti-inflammatory activities

of *L. johnsonii* N6.2 along with its ability to modulate the host immune responses may explain the mitigation of type 1 diabetes in BB-DP rats when fed daily with this bacterium [15, 83].

3. Probiotic effects on a master regulatory pathway

3.1. mTOR: a master regulator of major cellular functions

Like any living thing, a cell's main goal is to grow, proliferate, and ultimately, survive. This requires the coordination of multiple environmental signals, working synergistically through several pathways in order to culminate into a common outcome. Intricate organization and intracellular crosstalk is necessary for this to be accomplished. Often, these coordinated signals require a regulator to ensure that these functions are carried out efficiently. For most of these processes, the mechanistic target of rapamycin (mTOR) could be considered that important moderator. mTOR is a serine/threonine kinase that presents itself into two distinct complexes: mTORC1 and mTORC2. Ultimately, this pathway integrates external and internal cues to encourage a cell to grow, proliferate, and survive. It senses a diverse set of nutritional and environmental stimuli, including growth factors, amino acids, energy levels, oxygen and stress in order to stimulate anabolic cellular processes like protein and lipid synthesis, and to discourage catabolic processes like autophagy. Deregulation of this pathway has been heavily linked to metabolic disorders and cancer [84].

3.2. The mTOR complexes: mTORC1 and mTORC2

As of today, mTORC1 is better characterized out of the two mTOR complexes. This complex is composed of three core proteins and two inhibitory proteins as follows: mTOR, Raptor (regulatory protein associated with mTOR), mLST8 (mammalian lethal with Sec13 protein 8), DEPTOR (DEP domain containing mTOR interacting protein), and PRAS40 (proline-rich AKT substrate of 40kDA) [85]. A popular path to mTORC1 activation is through PI3K/AKT [86]. Here, growth factors and hormones bind to their receptor and activate the intracellular phosphatidylinositide 3-kinase (PI3K) which, through multiple interactions, leads to phosphorylation and partial activation of protein kinase B (AKT). AKT activation phosphorylates and consequently inhibits the tuberous sclerosis complex (TSC). This inactivation stimulates mTOR by inactivating Rheb's (Ras homolog enriched in brain) GTPase domain so that active GTP-bound Rheb binds to mTOR.

Activation of mTORC1 leads to an increase in protein synthesis, lipid biosynthesis, and a decrease in autophagy [85]. Downstream, mTORC1 promotes protein synthesis essentially through two main effectors: p70S6 kinase 1 (S6K1) and eIF4E binding protein (4EBP). S6K1 can also influence lipid biosynthesis by activating the sterol responsive element binding protein (SREBP), which promotes the transcription of genes involved in fatty acid and cholesterol biosynthesis [87]. However, this transcription factor can also be activated by mTORC1, by inhibiting Lipin1, a protein the keeps SREBP localized to the cytoplasm [88]. Peroxisome proliferator-activated receptor γ (PPARγ), a main regulator of adipogenesis, is also activated by

mTORC1 [89]. Autophagy is inhibited by mTORC1 through the inhibition two main effectors: ULK1 and DAP1 [90]. ULK1 is a kinase that forms a complex with other proteins required for autophagosome formation while DAP1 directly negatively regulates autophagy.

Even though mTORC2 still holds many secrets, we do know a bit about the complex and the functions it regulates. Along with mTOR itself, this second mTOR complex also contains mLST8 and DEPTOR. However, instead of Raptor, mTORC2 contains Rictor (rapamycin-insensitive companion of mTOR), mSIN (mammalian stress-activated map kinase interacting protein 1) and protor 1/2 (protein observed with Rictor 1 and 2) [85]. While mTORC1 is known to be affected by many external stimuli, mTORC2 is resistant to nutrients but is affected by growth factors through a mechanism requiring PI3K. Though this mechanism is poorly understood, it may require the use of ribosomes as ribosomes are needed for mTORC2 activation via a PI3K-dependent process [91].

Not much is known about mTORC2 activation and downstream studies do not hold many answers either. It does seem to primarily control cell survival and proliferation. It is known that when mTORC2 is activated it phosphorylates and fully activates AKT by phosphorylating at serine473 [92]. This mTORC2-dependent phosphorylation unlocks the AKT functions of inhibiting transcription factors FoxO1/3a, which regulates energy metabolism and apoptosis [93]. However, this phosphorylation is not required for AKT inhibition of the TSC complex, therefore mTORC1-dependent functions are not affected. mTORC2 can also directly phosphorylate SGK1, a kinase that controls ion transport and also inhibits FoxO1/3a [94]. Lastly, mTORC2 also regulates cytoskeletal dynamics through the activation of paxillin, PKC-α, and Rho GTPases, ultimately affecting cell shape and migration [95].

3.3. mTOR deregulation in disease

Since the mTOR pathway is heavily involved in functions affecting survival and growth and responds to growth factors, energy status, amino acids and oxygen, it is not at all surprising that deregulation of this pathway can cause serious systemic problems. Indeed, mTOR is a very complex pathway that seems to play a central role in many fundamental cellular processes. Since new mechanisms of action and regulation are constantly being discovered, it seems that this pathway still has many secrets to be told. Due to the importance of the functions mTOR controls, it is extremely important to keep this pathway in-check. Certainly, this pathway does contain intricate negative feedback loops and inactivating enzymes to prevent the pathway from going into a chronic state of activation. However, like all well-organized systems, a simple flaw could wreak havoc on the system, and there have been plenty of cases reported in disease and research of the consequences that occur in these circumstances.

Since the mTOR pathway integrates glucose homeostasis and lipid synthesis, it is not difficult to believe that disruptions in this pathway can lead to serious metabolic diseases. Indeed, mTOR has been heavily involved in obesity-related comorbidities, such as type 2 diabetes. A high fat diet, a contributing factor to these diseases, has been known to raise insulin, amino acids, and pro-inflammatory cytokines levels, which can affect mTOR activity. Type 2 diabetes occurs when cells become immune to insulin, even when sufficient insulin levels

accumulate to signal cells to take up glucose. mTORC1 has been implicated in regulating the insulin-producing pancreatic β cell function, as β cell-specific TSC component knockout mice revealed that young mice experienced increased β cell mass coordinated with higher insulin levels and increased glucose tolerance [96]. However, as the mice aged, these observations reversed, resulting in a decline of β-cell function over time [97]. This biphasic display could be explained through the feedback inhibition of insulin/PI3K/AKT by constitutive S6K1 expression [98]. At first, constant mTORC1 expression improves β-cell function, however this constant activation eventually accumulates in the S6K1-mediated inhibition of IRS1 upstream of mTORC1. Decrease in β cell function is also observed when mTORC2 signaling is knocked out. In this case, activation of AKT does not occur, which encourages FoxO1 activation. This causes a defect in glucose metabolism, leading to glucose intolerance due to a reduction in β-cell mass and proliferation, affecting insulin production and secretion [99]. It is clear that mTOR is a major regulator in β-cell viability and insulin signaling. Deregulation of this pathway has a great potential to cause insulin resistance leading to diabetes onset.

mTOR signaling also plays a significant role in obesity and non-alcoholic fatty liver disease, both of which can be characterized by an increase in adipogenesis. Fat, the most important energy storage site, accumulates in an mTORC1-activated state, while loss of mTORC1 results in leanness and resistance to high fat diet-induced obesity through enhanced mitochondrial respiration [100, 101]. This is because downstream effectors of mTORC1, 4E-BP and S6K1, regulate adipogenic transcription factors and their translation [102, 103]. Loss of mTORC2, on the other hand, results in impaired glucose transport in response to insulin stimulation and increased lipolysis translating to an escalation in circulating free fatty acids and glycerol [104]. Proliferation of adipose tissue is recognized to be the highest risk factor in developing obesity-related diseases. Over-activation of mTOR has been heavily connected in the tissues of obese and high fat diet-fed animals and its regulation is critical in maintaining a healthy state.

The liver is a multifaceted organ. Not only does it filter and detoxify the blood, it also produces and stores compounds utilized by the whole body. Of importance to this discussion, the liver is responsible for producing triglycerides, cholesterol, and ketone bodies that peripheral organs use as an energy source in low nutrient states. Like adipose tissue and pancreas, mTORC1/S6K1 activity in the liver is high in obese or nutrient dense states, leading to feedback inhibition of IRS and insulin resistance. This inhibition leads to the hyperglycemia and hyperinsulinemia characteristic of type 2 diabetes and insulin resistance. Interestingly, in the liver as well as other tissues, insulin loses its sensitivity yet still retains its ability to stimulate fatty acid synthesis. This could be explained by the fact that FoxO1 in primarily responsible for glucose metabolism in an mTORC2-dependent process, while mTORC1 promotes lipogenesis and this is primarily controlled via SREBP expression [105, 106]. Therefore, this may promote the double-edged sword of glucose intolerance and the stimulation of lipogenic processes, leading to obesity and insulin resistance in an mTOR-dependent manner.

Lastly, imperfect mTOR signaling plays an important role in many cancers. This pathway is made up of many proto-oncogenes and tumor suppressors that, if affected, could turn a cell into a constitutively growing and proliferating state characteristic of cancers. PTEN (phosphatase and tensin homolog) antagonizes the actions of PI3K, which phosphorylates phosphatidylinositol

4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PTEN recycles PIP3 back to PIP2, blocking downstream PI3K/AKT/mTOR signaling. Mutations in this gene blocks the ability of PTEN to recycle PIP3 and deactivate downstream effects, and it has been found to be frequently mutated in human cancers [107]. Another tumor suppressor, TSC1/2, suppresses chronic mTOR activation but, when mutated, can lead to abnormal and unregulated growth [108]. Hyperactivation of mTOR can also happen at the genomic level, as mutations in *MTOR* have also been found in various cancers [109]. Other oncogenic genes, such as *Akt*, *Pi3k*, and *Rheb*, have been described to encourage proliferation and tumor progression. Many of the pathways genes and proteins have been credited with encouraging a proliferative state when manipulated, and since the mTOR pathway is so vast and largely unknown, pinpointing the problem becomes an impossible feat. Even more challenging is finding treatments that are effective and do not have downstream adverse effects.

In response to stimulatory signals, such as insulin and nutrients, the combination of increased adiposity and insulin resistance resulting from chronic mTOR activation is the main driving factors contributing to metabolic disease. To further complicate the scene, genetic mutations or aberrantly functioning proteins can force a cell into a constitutively growing and dividing unit, reminiscent of cancer. Cancer and metabolic disorders are some of the most common diseases in modern times. To contain or prevent the occurrences of these diseases are the topics of many current research and clinical trials. The mTOR pathway coordinates cell growth and environmental conditions through an intercalated network that must adapt to unstable conditions. The complexity of this pathway, the diverse signals it recognizes, and the importance of the functions it regulates makes this a promising, albeit cumbersome, target for therapeutic intervention.

3.4. Therapeutic probiotic strategies to modulating mTOR

Although no studies have directly explored the interaction of probiotics with the mTOR pathway, it is likely to surface soon. With the microbiome a popular topic in research in relation to disease onset and now the emergence of mTOR as a main regulator of essential cellular functions whose deregulation is indicated in disease, it would be not all too surprising if a connection could be made between the two. To be able to implement a non-invasive strategy to treat diseases such as cancer or type 2 diabetes would be a huge leap forward in medical technology. Indeed, a main goal in microbiome research is to be able to understand the effects of these microorganisms in the gastrointestinal context, and to dissect their interactions with the host and their environment, including other microbial species and luminal contents. Though many groups have reported on some of the effects of specific microbial species, there is still much left to be discovered. Here, we will consider some of the connections these effects may have with the mTOR pathway (**Figure 3**), and discuss the potential consequences this may have on the host.

In many cases, disease onset is preceded by a systemic inflammatory response. This is also the case in cancer and metabolic disorders. As mentioned, inflammatory cytokines, such as TNFα, are known to be potent inducers of mTOR activity. TNFα is known to inhibit TSC1 through its activation of IKKβ, a link that has also been exposed in tumor angiogenesis [110]. A significant elevation of pro-inflammatory cytokines has also been described to be associated with metabolic disorders, such as obesity and type 2 diabetes. As is the case with cancer,

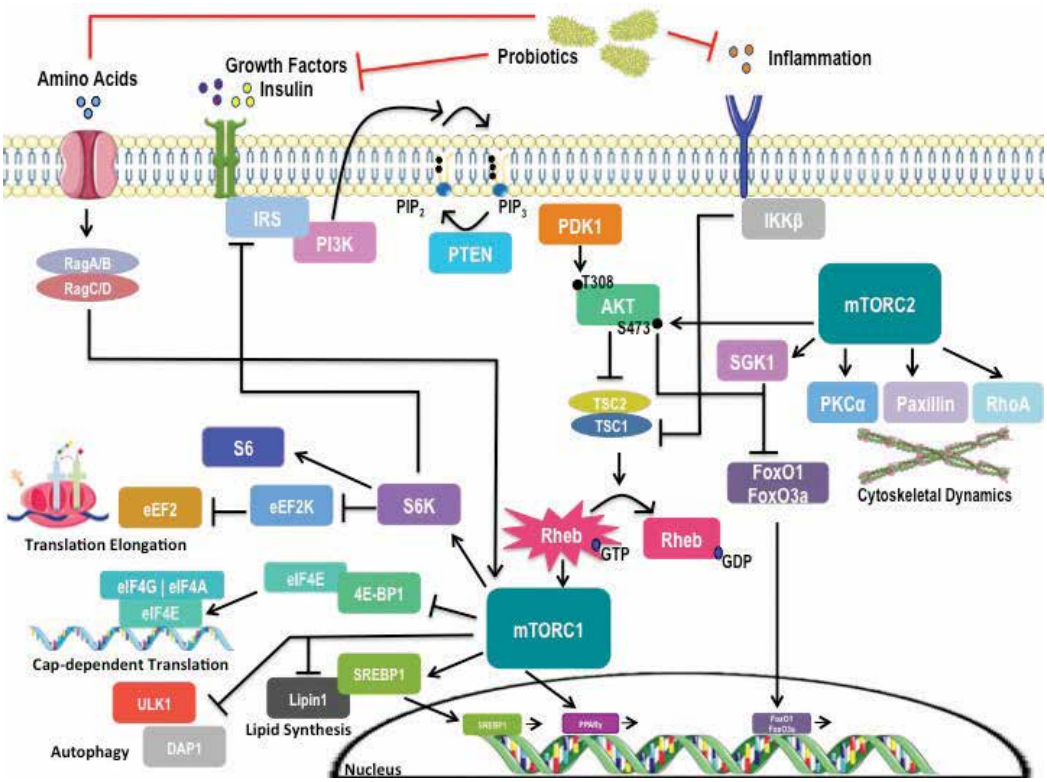

Figure 3. A simple representation of the mTOR pathway and some potential probiotic targets. External signals such as inflammation, growth factors, insulin, and amino acids can stimulate mTOR activity through a cascade of upstream effectors. These can activate processes that are required for a cell to grow, proliferate and survive. Using probiotics to target some of these stimulatory signals or enzymes within the pathway can help modulate its effects regarding disease onset.

IKKβ seems to also link inflammation to obesity-induced insulin resistance, and its inhibition could potentially be used to treat insulin resistance [111]. Coincidentally, numerous studies on probiotic strains have focused on alleviating inflammation and have even reported this to be correlated with reduced disease onset [15, 112]. Reducing the circulation of inflammatory cytokines will be less effective in activating IKKβ and therefore stimulating mTOR activity. Therefore, the successful alleviation of inflammatory cytokines with probiotics has the potential to reduce the activation of mTOR and its downstream effects, potentially reducing the incidence of modern diseases associated with chronic mTOR activation.

Insulin resistance occurs when the hormone insulin is insufficient in triggering cells to take in glucose to be converted into energy. Although insulin is produced at reasonable levels, glucose cannot enter the cells and therefore builds up in the blood, leading to hyperglycemia. Both insulin resistance and hyperglycemia are characteristic of type 2 diabetes, and it has been explained that chronic mTOR activation can contribute to this through the negative feedback loop connecting S6K1 to IRS. Obesity and a poor diet have also been described to be risk factors for type 2 diabetes, and associated with mTOR activity. Since the occurrence of type 2

diabetes is dramatically increasing and it continues to be one of the most prevalent diseases threatening human health, there have been many studies commenting on probiotic intervention to reduce symptoms of type 2 diabetes. Several *Bifidobacterium* and *Lactobacillus* strains are described to reducing weight gain, improving insulin-glucose homeostasis and overall improving metabolic syndrome in obese or high fat diet-fed mice [113, 114]. Even a study on a probiotic yeast was found to reduce metabolic syndrome symptoms and hepatic steatosis in obese and diabetic animals [115]. Clinical studies are now investigating this relationship and have reported improved insulin resistance in high fat, over-fed circumstances [116, 117]. However, these studies have rarely looked directly at the mechanism in which these probiotics contribute to human health, and even less often have any investigated into the effects on mTOR. Needless to say, it is possible that these mechanisms could be mTOR-mediated, however more work into this area is needed.

One of the many benefits that our microbial symbionts provide for us is the ability to produce or release substances that our bodies are not capable of doing itself. These substances include vitamins, antimicrobials, butyrate, and other short chain fatty acids (SCFA). In fact, even the famous inhibitor in which the pathway is named after, rapamycin, is produced by the bacterium *Streptomyces hygroscopicus*, providing more evidence that microbes can make specific ligands that interfere with the activity of mTOR enzymes. Studies have elucidated the beneficial effects of butyrate have on colon diseases, such as ulcerative colitis, Crohn's disease, and cancer [118]. To date, two main SCFA signaling mechanisms have been described: the inactivation of histone deacetylases (HDAC) and the stimulation of G-protein-coupled receptors. One study has even uncovered the role of HDAC-activated S6K1 in promoting immune tolerance through T-cell differentiation into effector and regular T cells due to SCFAs [119]. This response is important when cells are faced with a potent stimulus. Instead of over reacting to the stimuli, the T cells emit tolerant signals to be able to neutralize the threat instead of creating a systemic inflammatory response. Additionally, some probiotic strains encode for unique enzymes that can cleave off phenols, or natural antioxidants, from dietary fiber [17, 120, 121]. Coincidentally, many of the inhibitors of the mTOR pathway, such as the popular rapamycin and its derivatives, are cyclic and phenolic in nature. This opens up a new avenue of research, exploring natural food components released by probiotics in controlling pathways whose deregulation is associated with diseases. Few studies have explored this area, but one group discusses the ability of cranberry proanthocyanins to encourage autophagy in esophageal adenocarcinoma cells via inhibition of the PI3K/AKT/mTOR pathway [122]. Another phenolic compound isolated from a shrub is described to disrupt mTORC1 complex and activate the AMPK/TSC signaling cascade, preventing breast tumor growth [123]. Since mTOR activity is aggressive in tumor development, preventing its bodily dissemination through natural food components seems like a far less intrusive procedure than current cancer therapies. Lastly, bacteria can alter the bioavailability of amino acids through their natural metabolism. They can utilize host-derived amino acids, provide amino acids to the host, or disrupt host pathways involved in amino acid digestion or synthesis [80, 124]. Amino acids, particularly arginine and leucine, are essential for mTORC1 activation [125]. Commensals in the intestine have been reported in utilizing these amino acids for protein synthesis, thereby limiting their availability for host-sensitive pathways [126, 127]. However, amino acid producing bacteria within the human intestine can contribute to this available pool of amino acids [128]. The homeostatic maintenance of the bioavailable

pool of amino acids by the gut microbiota may be an important modulator of mTOR activity *in vivo*, thereby controlling disease development. Still, with the emergence of new mTOR data, we are finding that the list of potential inducers of mTOR to be very extensive. Although arginine and leucine are deemed the most important inducers of mTOR, other amino acids have been found to be able to trigger this pathway, and bacteria that have the ability to disrupt host biochemical pathways can regulate this expression [80, 129]. The complexity of the microbial-host relationship in the context of communal metabolites provides an intricate insight into the regulation of important regulatory pathways.

The reduction of mTOR through pharmaceutical intervention has also been a popular area for research. One drawback to this method is that these techniques aim to directly inhibit this pathway through contact with its key mediators. Although, this seems like the simplest and most effective way to prevent mTOR-mediated disease onset, it could create drastic effects. Since this pathway focuses on essential cellular functions, total inhibition of this pathway could do more harm than good. As these drugs are sometimes not natural chemicals, they can also induce unrelated but potentially critical side effects in the body. The best method of action may be to focus on indirect approaches to modulate mTOR activity, rather than trying to completely prevent its activation. These indirect methods could come in the form of moderating its stimulatory signals, such as inflammation and insulin. As we discover more of the health benefits probiotics have to offer, it is clear that this is a multifaceted interaction with the host. After all, these are living organisms, consuming, excreting, and doing what is necessary to survive rather than a chemical that has no consideration of its existence. It is possible that this complex relationship could be what we need to keep our body in balance. Therefore, the answers to relieving some of today's most aggressive diseases could come from our own microflora.

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Conflict of interest

Authors declare no conflicts of interest.

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Chapter 3

Probiotics and Its Relationship with the Cardiovascular System

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Additional information is available at the end of the chapter

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Abstract

Cardiovascular disease is a major health issue worldwide. Individuals who have cardiovascular disease, are often at risk or already have other diseases, which together can lead to metabolic syndromes and possibly increase the risk of morbidity and mortality. Gut microbial balance is increasingly being recognized as a possible risk factor in cardiovascular illnesses. Studies published so far have shown a possible link to hypertension, hyperlipidemia and associated cardiac illnesses. Balance of the colonic flora seems to improve these co-morbid conditions. Probiotics have been studied in several studies to determine if their use provides a beneficial non-pharmacological treatment option for diseases such as diabetes, obesity, hypercholesterolemia, hypertension, chronic kidney disease, cardiomyopathy and atherosclerosis. Placebo, double blinded controlled studies are s needed to determine if these perceived beneficial effects exists and to what extent probiotics play in the overall outcome in cardiovascular diseases.

Keywords: probiotics, cardiovascular health, hypertension, obesity, diabetes mellitus

1. Introduction

Cardiovascular disease (CVD) is a major cause of death worldwide. There are disease-associated risks that can be either modifiable or unmodifiable factors and examples are low-density lipoprotein (LDL) cholesterol, increased triglyceride-rich lipoproteins, and low levels of high-density lipoprotein (HDL) cholesterol [13]. An individual's personal gene makeup, body composition, health, and having certain preexisting disease states can also influence their risk of having a CVD. These factors often contribute to a group of conditions leading to metabolic

syndrome. Metabolic syndrome increases an individual's chance of having a disease such as CVD and/or diabetes.

Gut microbes are thought to be responsible for healthy outcomes in terms of the gastrointestinal (GI) tract, as well as positive health benefits distant to the GI tract. The alteration to dietary macronutrient ingestion has increased the prevalence of metabolic disorders which has been shown to be related to microbial imbalance to the gut as part of the pathogenesis [7, 9]. A meta-analysis of several studies found about 1100 bacteria species and their related properties in relation to diseases such as diabetes mellitus, cardiovascular disease, obesity, and cancer [7]. The change in gut microbe related to a disease state can often be associated with an individual's diet. Diets that are high in fat and/or sugar and low in fiber have a negative effect on gut ecosystem [18]. Therefore, diet modification to alter the composition of gut bacteria is vital for either prophylaxis or the treatment of some diseases. This makes gut microenvironment a focus point in the prevention of unhealthy state and improvement to a healthy state in order to avoid metabolic syndrome-related diseases such as cardiovascular disease and diabetes.

Modifying gut microbiota with probiotics has been in practice for centuries and is now being studied in relation to treatment and/or prophylaxis for metabolic syndrome and related diseases such as cardiovascular disease [9]. The more recent metagenomics studies are those that have demonstrated that probiotics are involved in host immune modulation and influence the development and physiology of organs. Therefore, they have been identified as the possible medical therapies to treat GI disorders and to restore an impaired gut ecosystem [8]. Studies have strengthened the idea of the importance of probiotics in aiding the prevention and prophylaxis of gut disorders, urogenital, and respiratory infections with their results [8]. The hypothesis that they could aid in the fight against metabolic disorders is based on them having an effect on the modulation of composition and function of interstitial microbiota [7]. Background information on probiotics, as well as current studies that have observed the gut microbiota in cardiovascular disease-related conditions such as obesity, diabetic, hypercholesterolemia, hypertension, cardio-arterial disease, and cardiomyopathy based on past studies, has been analyzed.

2. Probiotics

Individuals using probiotics for the improvement of health have a well-known history, especially lactic acid bacteria (LAB) and *Bifidobacteria*, as well as prebiotics as part of food or fermented food [7, 20]. Dating back to 76 BC, there was the recommendation of ingestion of fermented milk products for those who had gastroenteritis [7]. The idea behind probiotics being used as a way to alter interstitial microbial balance started in the twentieth century from the work of Metchnikoff [7, 9]. With new advance techniques such as DNA-based analyses, there have been a significant number of research that observed different bacteria and their properties that are related to both positive and negative influences on the human body in the disease and healthy state [7]. The following has been stated to be important in probiotic research: identification, maintenance, and characterization of probiotic strains in live conditions, so potency is preserved, and they arrive alive in a state of action which varies [25].

Probiotics have been observed to have a positive effect on gut microbial and are often studied in a combined relationship with prebiotics termed symbiotic.

Some examples of the positive outcome from probiotics have been an improved immune system through immunoglobulin production, trigger cell-mediated immune response, and help in the treatment of gut disorders such as irritable bowel syndrome (IBS), *Clostridium difficile* colitis, gastric ulcers lactose intolerance, and antibiotic-associated diarrhea (AAD). Positive results from probiotics are caused by the multifactorial process related to them that results in the production of organic acids, hydrogen peroxide, bacteriocins, bacteriocin-link inhibitory substances, short-chain fatty acid (SCFA)-conjugated linoleic acid, and ϒ-amino butyric acid [8]. These can cause improvements that range from improved bone density, anxiety, hyperammonemia, and improved blood lipid profile to name a few [3]. This is based on the proven functions of probiotics such as balancing intestinal microbiota, modulating the immune system, and exerting metabolic influences [4].

Prebiotics often provided with probiotics can contribute to the influences on the bacteria population in the human gut [7, 9]. Prebiotics is a type of nondigestible fiber compound, which is able to bypass the upper gastrointestinal tract, remain undigested, and reach the colon where they are fermented by the gut microflora. It is a type of food source for probiotics (microbiota) and it regulates the growth and activity of gut microbiota, resulting in an improved gut health and strengthened immune system [7, 14]. In order for prebiotics to provide a beneficial role, they must have the following three characteristics: "resistance to gastric acidity, hydrolysis by mammalian enzyme, and gastrointestinal absorption, fermentation by intestinal microflora and selective stimulation of growth and/or activity of intestinal bacteria associated with health and well-being." Various chain-length oligosaccharides are the most common that are studied and those include fructo-oligosaccharide and galacto-oligosaccharide/transgalacto-oligosaccharides [2, 7]. Tri-, di-, and some monosaccharides may also be used as prebiotics if they have host-indigestible bonds.

Prebiotics mode of action is taking advantage of the commensals that are already in the host; they use this to degrade their otherwise indigestible bonds, which support the microbial survival [1]. They are used as fermented ingredients that induce the growth or activity of microorganism. *Bifidobacterium* and *Lactobacillus* have been identified for responding to the administration of prebiotics, for example, oligofructose (OFS) stimulates the growth of *Bifidobacterium*. Prebiotics beneficial properties are not just limited to the GI system. Once prebiotics have been selective fermentation, there will be an increase in the number of commensals while lowering other neutral/harmful organisms which support symbiotic gut microbiota composition. It has been shown that though the "gut-brain axis," some such as fructo-oligosaccharide and galacto-oligosaccharide are able to modulate neural growth factors such as brain-derived neurotrophic factors and synaptic proteins [2]. This can affect memory, attention, learning, and mood. When prebiotics and probiotics are used together, it is called synbiotics.

Fermented foods consist of microorganisms that are either functioning or nonfunctioning. One of the functioning actions is to stimulate probiotic function [6]. *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella* are lactic acid bacteria associated with fermented food along with species of *Bacillus*, *Bifidobacterium*, *Brachybacterium*, *Brevibacterium*,

and *Probacterium* [6]. *Lactobacillus* and *Enterococcus* are LABs, which with *Bifidobacterium* are the most commonly used probiotics. The most common traditional source of the probiotic *Lactobacilli* is fermented milk [6]. These microorganisms have several properties such as probiotic, antimicrobial, antioxidant, peptide production, fibrinolytic activity, poly-glutamic acid, degradation of antinutritive compounds, and ambrotose complex memory [6]. *Bifidobacterium* and *Lactobacilli* will selectively ferment prebiotics which cause an increase of these commensals while displacing other pathogenic or neutral organisms [2].

Probiotics containing a live microorganism should be used with caution in patients that are immunocompromised because they can cause infection or pathogenic colonization [27]. This has been supported by several studies. A study that observed renal-transplant patients with AIDS found that Lactobacillemia, which is not a common cause of bacteremia, occurred. Lactobacillemia was found in other patients who were immunocompromised with the following conditions: cancer, organ transplantation, diabetes mellitus, and recent surgery. Out of these patients, fever was presented in all of them and 15% developed sepsis but it is important to note that Lactobacillemia can have a wide range of clinical features. A probiotic consumed by a patient who had advanced and severe bicuspid aortic valve stenosis developed *L. paracasei* endocarditis. *Lactobacillus* may be under recorded because it is not observed as a pathogen and is also usually determined as part of a polymicrobial infection [33]. These are just a few cases in which an infection was caused, and overall, there have been studies that support the safety of probiotics consumed by groups of immunocompromised patients [33]. Probiotics can also affect some interaction with other drugs, for example, they could interfere with the production of vitamin K and therefore could affect the sensitivity to some drugs like warfarin [27].

Lactic acid bacteria tend to produce bioactive compounds, which are frequently found in fermented products due to LAB-elective habitant food, especially in diary. Biogenic amines are the main health risk in fermented food [5]. These compounds can sometimes cause allergies, hypertensive crises, and headaches. Also, it is important to make sure that probiotics, which are used to aid in the control of LDL levels, do not affect cardiac myocyte function, increase fat deposition, or cause cancer [20]. Cancer is a risk because secondary bile salts may disrupt DNA repair pathway. This disruption can lead to oxidative stress in epithelial cells which can start tumor formation [21]. These adverse effects on human are usually not a concern in generally healthy individuals [6].

3. Relationship of CVS to probiotic use

3.1. Obesity

Obesity, which causes a low-grade inflammation, is a risk factor for cardiovascular disease, diabetes, dyslipidemia, premature death, hepatobiliary disease, and several cancers. There is an estimate of 1.7 billion people in the world that are overweight. Obese individuals tend to have an altered composition of intestinal microbiota, which suggest that intestinal microbiocenosis can be considered the environmental factor that creates the development of obesity

[5]. Some of the effects of altered bacteria composition in the gut is linked to obesity due to several changes such as downregulated activity of FIAF and AMK, impaired production of SCFAs, increased inflammation, altered LPS-endocannabinoid (eCB) system regulatory loops, and bile acid metabolism [5]. The cause of the alteration is believed to be linked through the host's diet. An example of this is that there was a reduction in *Lactobacillus* and *Bifidobacterium* and was observed in mice when they consumed high-fat diets [5]. This change of environment is the basis of studies that have shown that gut microbiota plays a role in energy homeostasis and bodyweight, therefore affecting the pathophysiology of obesity [5, 18].

In obese individuals, the different microorganism environment is believed to affect adiposity and alter the regulation to fat storage [7]. Insulin-type fructan affects the gut ecology and stimulates immune cells leading to a decrease in the weight gain and fat mass in obese individuals [7]. In a meta-analysis, several studies found that there was an increased prevalence of *Firmicutes* shown with obesity phenotypes. These bacteria interfere in a negative relation with metabolism and insulin sensitivity [26]. Probiotics, while resulting in more subtle effects in humans versus mice studies, are now being studied as a way to modulate gut microbiota in relation to obesity [20]. This is because certain traits in probiotic cultures such as exopolysaccharides, CLA, and GABA production were found to have a positive effect on host lipid metabolism and gut microbial composition [8].

An increased number of *Lachnospiraceae* family in obese female microbiota were altered when probiotics containing *L. rhamnosus* CGMCC1.3724 (which reduces *Lachnospiraceae* family) were administered along with an energy-strict diet [38]. However, the probiotics that were related to fat mass in Ref. [38] also caused a decrease of leptin levels, which may lead to a need for the supplementation of leptin in order to maintain weight loss. Probiotics and weight loss have also been linked to a decrease in ghrelin, which could assist in maintaining the weight loss even with the loss of leptin [41]. When *L. gasseri* strain was given in fermented milk for 12 weeks, there was a decrease in abdominal visceral fat in adults with large visceral fat areas [18]. Supporting this, there have been other studies that when looked at the outcome of probiotic consumption, there was a decrease in both body mass index (BMI) and waist circumferences [13]. However, those were a limited number of studies, and additional studies are required, including those that will observe the effect of probiotics on energy balance-related hormones.

In multi-strain probiotic therapies, with 8 weeks of treatment, obese individuals showed a decreased weight, waist circumference, and serum cholesterol levels. This study also supported the idea that probiotics caused results not only by their own metabolism but through probiotic alteration of the gut microbe with an increase of *L. plantarum* population and other Gram-negative bacteria [20]. When prebiotics were added, there was control of overexpression of several host genes that have been known to be related to both adiposity and inflammation [27]. However, the altered level in gut microbe with one probiotic only had a subtle effect, and more studies are necessary to understand if and what probiotics provide a change to obese individual's gut microbe.

Gut bacteria also play a role in obesity through the regulation of inflammation. The relation between low-grade systemic inflammation and obesity is weakened through peptides produced in the gut. These peptides' synthesis is affected by the composition of gut microbiota [7].

An example of this is the serum amyloid A3 protein where the expression in adipose tissue is regulated by gut microbiota [7]. Any alteration to the gut microbiota could then also potentially play a role in body weight due to intestinal microbiota effects on adiposity and the regulation of fat storages.

3.2. Diabetes

Having diabetes or having the risk of diabetes is often associated with a higher risk for cardiovascular disease. This is because of a compensatory action resulting in hyperinsulinemia which leads to a variety of metabolic abnormalities. Individuals who have diabetes were found to have altered intestinal microbiota which can cause increased adiposity, B-cell dysfunction, metabolic endotoxemia, systemic inflammation, and oxidative stress related to their disease. SCFA is an important function in type 2 diabetes mellitus (T2DM); however, bacteria producing SCFA numbers are lower in diabetic individual [7]. Probiotics may offer a beneficial therapy for diabetic patients through increasing SCFA and other methods.

Oral supplements, which contained viable and freeze-dried stains, were found to reduce fasting plasma glucose when compared to a placebo group. Fermented food was also noted to not only aid in the prevention of diabetes but also cause favorable changes in those already diagnosed with diabetes [6]. This could be due to some probiotics delaying the glucose intolerance and hyperglycemia state in individuals. For those with diabetes, some probiotics in fermented food decrease insulin requirements and could increase insulin sensitivity for non-diabetics [6].

Diabetes has a connection to long-term inflammation. This is due to the consumption of high fats and high fructose which causes chronic inflammation leading to the induction of insulin resistance (IR) and disruption of gut flora. This is supported by studies, which have found that certain diets, for example, high-fat diets, tend to increase lipopolysaccharide (LPS) contained in gut microbiota which leads to a decrease of *Bifidobacteria*. This leads to an inflammation state which may be associated to insulin resistance and weight gain [1]. Different probiotics have differential immune pro- or anti-inflammatory action through the attenuation of nuclear factor kappa B (NF-kB) [4]. *Lactobacillus* is a lactic acid bacteria that contains immune stimulating properties [28]. Probiotics, *L. reuteri,* and *L. plantarum* have anti-inflammatory and antioxidant effects which can aid in the management of diabetes [9]. For example, C-reactive protein (CRP), an inflammation marker, is noted to decrease when these probiotic supplements are used [13].

In a meta-analysis, there was no statistically significant glucose-lowering effect of probiotics when combined with prebiotics [1]. However, prebiotics may affect the inflammation state due to prebiotics having immunomodulatory benefits. In a study, prebiotics were found to alleviate chronic inflammation, which could lower the risk of development of cardiovascular disease and diabetes [2]. Probiotics may even possibly assist in the prevention of diabetes through bacterial translocation to mesenteric adipose tissue. This is mediated through acetate production and an increase in gut epithelial integrity [26].

Hyperglycemia, which is a property of diabetes, is a term given when a person has continuous high-fasting blood glucose (>6.1) and is associated with different diseases, the main one being

diabetes mellitus [1]. The first line of treatment is proper nutrition and physical activity [1]. Probiotic supplements along with prebiotics were found to improve the hyperglycemia state. When multi-strain probiotics along with symbiotic supplements were provided to individuals in a hyperglycemia state as their baseline, there was an improvement in their blood glucose level (BGL) [1]. Glucose tolerance and increased satiety with weight loss were found when individuals were administered OFS which lead to *Bifidobacterium* and endotoxin levels to be normalized. Butyrate, which has properties of propionate that can lower blood glucose, is produced by several bacteria [4]. Other studies found that with a symbiotic shake of *L. acidophilus*, *Bifidobacterium* and *L. rhamnosus* caused a 38% decrease in blood glucose levels for patients with T2DM. Though these studies demonstrate that supplementation with probiotics with symbiotic may help in the control of hyperglycemia and T2DM, larger studies are needed to confirm. The glucose-lowering effect is due to the metabolites of these bacteria which was shown to affect biological signaling pathways, modulated genes involved in ubiquitination and proteasome process, and altered autonomic nerve activity [1]. It is also vital to note that probiotics or synbiotic alone did not cause a significant reduction in fasting blood glucose levels.

3.3. Hypercholesterolemia

Cardiovascular disease, affecting both blood vessels and/or heart, usually is the result of hypercholesterolemia and dyslipidemia. There have not been direct studies that compare the effect of prebiotic intake on cardiovascular health; however, there has been an observation on the serum lipid profiles, which all have an effect on CV [2]. These experiments have observed the effect of probiotics and/or prebiotics both in vitro and in vivo on lowering cholesterol [7]. In order to use probiotics to help lower cholesterol, the probiotics adhesion property to the human intestinal epithelial cells is a critical characteristic that must be considered [14]. This characteristic is to ensure that there is extended probiotic transit time in the gastrointestinal trace which was found to cause cholesterol-lowering effects in vivo.

Studies have shown a lower low-density lipoprotein and total cholesterol, along with increases in high-density lipoprotein cholesterol, a reduction in systolic blood pressure (SBP), increases in antioxidant activity, and influences on leptin regulation as a result of probiotics [9]. This is done through an enzyme called bile salt hydrolase (BSH) which causes a decrease in the absorption of cholesterol in the blood stream and is an essential criterion for the selection of probacteria [9, 13]. This enzyme unconjugated bile acids, which eventually cause a decrease in circulating triglycerides and plasma LDL and VLDL levels [12, 20]. The most associated BSH active probiotics are *Lactobacillus*, *Lactococcus,* and *Bifidobacterium* [21]. These bacteria have been observed to lower cholesterol both in vitro and in vivo [28].

For example, see [14], which found that *L. fermentum* NCIMB 5221 and NCIMB 2797 were able to lower cholesterol in an in vitro analysis. They found that *L. plantarum* ATC 14917 had the best results [22]. Another study found that the BSH candidate *L. reuteri* NCIMB 30242 had the capabilities to lower cholesterol in otherwise healthy individuals [12]. This is because *Lactobacillus* species are able to colonize and survive in small intestines [21]. These studies have demonstrated why lactic acid bacteria with BSH are being classified as having hypocholesterol effect. More specifically, trials that used multiple strains versus single strains and fermented products

versus capsule found that multi-strain and fermented methods both caused a decrease in total cholesterol and LDL [13].

Probiotic soy products in association with cardiovascular risk factors were observed. The fecal microbiota that was used was *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Enterobacteriaceae*, and *Clostridium* spp. populations. Their results showed a negative correlation with *Enterococcus* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. with cholesterol, non-HDL cholesterol, and autoantibody against LDL [29]. However, this study was performed with rabbits, and future studies with human subjects are necessary for a confirmed effect.

High-density cholesterol, HDL, is considered good cholesterol and is important for removing "bad" cholesterol from the blood stream. This study found that there was a positive correlation between *Lactobacillus*, *Bifidobacterium*, *Enterococcus,* and HDL-C levels [29]. However, in relation to T2DM patients, there were some studies, which found that probiotics failed to maintain a significant effect on lipid profiles [7]. Prebiotics, however, were found to maintain hypocholesterolemic effects in the T2DM individuals [7].

Other methods in which probiotics affect blood lipids include binding and incorporating cholesterol to their cell membrane, which decreases the amount of intestinal cholesterol available for absorption, and by producing SCFA which inhibit hydroxymethylglutaryl CoA reductase. *Lactobacillus* species have protease-sensitive receptors on their cell surface. These receptors bind to exogenous cholesterol or phosphatidylcholine vessels, which then incorporate cholesterol into their cell membrane. This is strain- and growth-dependent action [21].

Probiotics, performing the mechanism of a 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, was shown with dietary fibers (prebiotics) altering the functionality of gut microbiome including the stimulation of microbial metabolite production such as short-chain fatty acid which impacts cholesterol metabolism. The lowering of cholesterol with prebiotics is believed to occur through two mechanisms. The first one is it lowers cholesterol absorption by enhancing cholesterol excretion via feces and the second is through the production of SCFAs upon selective fermentation by intestinal bacterial microflora. Inulin and arabinoxylan, both prebiotics, can alter gut microbiome to stimulate SCFA production which has been already shown to effect cholesterol metabolism [12]. The mechanism behind this is that cholesterol is removed though the incorporation of cholesterol into cellular membranes in the intestine [13].

In terms of fermented food, *Monascus purpureus* rice was found have similar actions as statin and acted as a HMG-CoA reductase inhibitor, decreasing the makeup of cholesterol [6]. The studies that have conflicting findings could possibly be due to the delivery system. Studies varied whether the probiotics were given in capsule versus fermented foods. However, in a limited number of meta-analysis of studies, it was found that probiotics using fermented foods were more effective in reducing total cholesterol and LDL than in capsule [13]. In the majority of studies that were reviewed, there was no control for individual's lifestyles in human subjects which could alter the findings.

3.4. Hypertension

Hypertension has several risk factors, such as sedentary lifestyle, lipid and hypercholesterolemia, chronic inflammation, inconsistent modulation of renin-angiotensin system (RAS),

sodium sensitivity, personal habits, anxiety, and stress. While dietary strategies have been the focus of target for repairing the disturbed gut microbiota, probiotics have been found to decrease systolic/diastolic pressures (approximately 14–6.9 mm drop) in prehypertensive and hypertensive patients. This blood pressure (BP)-lowering effect through probiotics is due to a decrease in nitrogen oxide production in macrophages, reducing reactive oxygen species and enhancing dietary calcium absorption using different mechanism. These mechanisms have been found to be related to the production of SCFAs, CLA, GA A, and angiotensin-converting enzyme (ACE) inhibitor peptides [8]. Short-chain fatty acids (SCFAs), which have a role in both energy metabolism and adipose tissue expansion, also have two sensory receptors that have been linked to BP regulation. Some of the probiotic strains that were noted to cause a decrease in SBP were *L. casei*, *Streptococcus thermophiles*, *L. plantarum*, and *L. helveticus* [9]. Fermented milk products have been shown to have antihypertensive properties in both animal models and clinical trials [6]. Blood pressure release may also be due to a decrease in blood lipids, body weight, and IR.

On continuing, blood pressure is normally controlled with a variety of biochemical pathways, including the RAS system. The generation of antihypertensive bioactive peptides causes an ACE-inhibitory activity [8]. Different strains of probiotics have varying potencies as ACE inhibitory activity based on different bioactive peptides [18]. When prebiotics were used along with probiotics or the probiotic strains were enhanced via fermentation substrates, the proteolytic activity and ACE inhibition were increased [20]. Fermentation is able to produce bioactive ACE-inhibitory type peptides, casokinins and lactokinins. Probiotics are able to generate these peptides though fermentation having caseinolytic and lactose hydrolyzing enzyme systems [9]. Consuming probiotic soy milk led to a decrease in BP in a limited number of type II diabetic mellitus subject in a clinical trial lasting 8 weeks [15]. This study did not find any alterations of anthropometric measures which had been found in other studies. This could be that there are strain-specific properties [15]. However, subpopulation studies showed no significant difference and there are no definitive recommendations at this time.

3.5. CAD

Cardio-arterial diseases are often associated with hypercholesterolemia, diabetes, and other metabolic-related diseases. Alteration to the gut microbiota can cause a detrimental risk of obtaining a cardio-arterial disease/state such as atherosclerosis. The change in gut microbiota can cause an increase in the level of trimethylamine N-oxide (TMAO), which has been linked to an increased risk of major adverse cardiovascular events observed in large clinical cohorts. However, additional studies are needed to determine the mechanism of CVD through TMAO [7].

Apo A-V deficient mice were found to have increased precursors of small dense LDL, which is a predictor of coronary artery disease [16]. This deficiency has been observed with bile salt hydrolase expressing probiotics to have an important role in not only lipid metabolism but also atherosclerosis development. *L. reuteri* NCIMB 30242 when provided to non-diabetic subjects with hypertriglyceridemia caused a decrease in apolipoprotein B, which is associated with atherogenic VLDL and LDL products [16]. It was also shown to reduce CRP and fibrinogen which are two factors of atherogenesis [12]. However, this study only included small healthy

hypercholesterolemia population, and the probiotic was given either in capsule or in yoghurt format. In mice, *Lactobacillus* species was found to lower arteriosclerosis [20]. When provided through powered supplement, *L. curvatus* and *L. plantarum* caused a significant increase in apo A-V [16]. With varying methods of providing the probiotics, more controlled studies are necessary to understand the relationship between probiotics and cardio-arterial disease.

Fermented products may provide a decrease in the development of atherosclerosis with the activation of G-protein-coupled bile acid receptor [25]. In a study that compared atherosclerotic lesions in the aortic vessel in animals treated with fermented soy product supplements versus a control group, the ones that were provided the supplement was found to have a lower percentage of aortic vessel covered with lesions [29]. Fermented whole grains are also able to lower coronary heart disease [6].

3.6. Heart failure

Heart failure causes a variety of systemic effects on multiple organs. While there are no heart failure changes observed to effect the gut microbial composition, there have been changes that could cause or increase the incidence of heart failures. New research is currently observing probiotics therapy providing direct cardio-protective effect to the heart. This protection would result in a reduced ischemic injury and improve cardiac function after an infarction [20]. TMAO, which is effected by gut microbiota, can be linked to both the development and progression of atherosclerosis and cardiovascular disease and is effected by gut microbiota [21]. However, a majority of studies have only observed the effects in mice. Continuing due to individuals not realizing that they are at risk for infarction, consuming probiotics as prophylaxis is unlikely and the prevalence of heart failure is stagnant.

3.7. Chronic kidney diseases

Patients with chronic kidney disease have an increased risk for cardiovascular disease through having hyperhomocysteinemia, increased lipoprotein, oxidative stress, and inflammation. Vascular dysfunction in both humans and experimental animals with CKD has been discovered to be due to an increased production and impaired renal excretion of p-cresyl sulfate and indoxyl sulfate which pairs CKD with vascular disease. These toxins along with others are normally cleared by the kidneys. When kidney patients were provided probiotics, there was a decrease in those toxins. However, due to the uremic environment of the gut that is often associated with CKD, probiotic may become ineffective or less ineffective [23].

3.8. Relationship to GI system

A population of microbes that assist the host's biochemical metabolic and immunological balance necessary for health maintenance is termed normal microbiota [6]. Both composition and function characterize the biodiversity of microbiota [4]. The gastrointestinal microbiota includes bacteria, archaea, protozoa, fungi, and different viruses, with anaerobic bacteria and the predominant source [4]. The numbers range from 10 to 100 trillion microorganisms in the GI tract, which, based on an individual's genetic age and diet, vary from individual to

individual [9]. From the time an individual is born until his/her death, there will be more than 500 different species of microorganism that are contained in the human body [6, 7]. An individual's microbial diversity changes throughout his/her life span and depends on a person's health-related interaction between gut microbiota and host's overall health [7]. There are even geographic variations that have been found in relation to the type of *Lactobacillus*, varying from the western and eastern hemispheres. The factors that can influence a person's microbiota are genetics, age, diet, and antibiotic use [8].

The colon has the largest variety of microorganism and is the focus part of most studies [4]. *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, and *Bacteroidetes* are the dominant species of obligate microflora [5]. Lactic acid-producing *Bifidobacterium* and *Lactobacillus* are often the focal points of studies due to their beneficial effects that is caused by their expression of immunomodulatory and pathogen-antagonistic molecules [2]. These bacteria produce butyrate, which highlights some properties of propionate and is observed as the preferred metabolic fuel for colonocytes possessing antineoplastic properties. This contributes to energy production [4]. Propionate affects colonic muscular contraction, relaxation of resistance vessels, and stimulation of colonic electrolyte transport and insulin resistance [4].

There are several ways that a human's microbiota aids overall health; examples of this include endogenous symbiont microorganisms, microbiota, changing not only gene expression but also have an effect on pH, redox balance, and the ratio between pro-inflammatory and anti-inflammatory cytokines. There are also studies which showed normal microbiota effect on brain metabolism, the immune system, and a couple of homeostatic routes [3]. There have been several studies that have noted a change of gut microbiota in several conditions/diseases such as obesity, fatty liver, insulin-resistant diabetes mellitus, and hypertension [11]. Some examples of these changes are an increase in *Firmicutes* and a decrease in *Bacteroidetes* [18]. With recent studies showing gut microbiota related to the pathogenesis of cardiovascular disease, probiotics, which are live microbial food supplements, could balance intestinal microbial resulting in the treatment or prevention of cardiovascular disease [9, 11].

The gut environment also plays a role in the type of bacteria found per location in the tract. The tract varies from an alkaline pH in the small bowel to an acidic pH in the stomach [31, 32]. Using the 16 s ribosomal RNA gene sequence-based metagenomics methods, it has been determined that 90% of bacteria of the gut belong mainly to the *Bacteroidetes* and *Firmicutes* phylum [27]. It has been discovered that both are lactic acid bacteria which are vital to the gastrointestinal track normal residents. These two are commonly used in fermented food for the prevention and treatment of different disorders ranging from constipation to high cholesterol levels [27].

When an individual is healthy, most of the microbiota act symbiotically with the host. The major metabolic function of microbiota is to assist with the harvest of nutrients and energy from different diets that human's consume [4]. The interaction between the gut epithelial cells and the microbes and the metabolites produced is responsible for the maturation of intestinal epithelial cells, enteric nervous system, intestinal vascular system, and the mucosal immune system. However, an imbalance in gut bacteria has been shown in numerous studies to be linked to a variety of diseases. Intestinal disease state can affect the microflora, impair the gut barrier, and/or cause intestinal inflammation which can all lead to imbalance in gut bacteria population

[31]. In order to reestablish a balance, probiotics, prebiotics, and synbiotics have been used and observed. Probiotics are able to affect the GI tract through their interaction with the intestinal epithelial cells, luminal flora, and mucosal immune cell components of the GI tract [28].

Antibiotics usage in early life has been determined to deplete some components of microbiota causing disrupted normal gut microbiota development [4]. Prebiotics such as fructooligosaccharides do not support the growth of antibiotic-related pathogens like *C. difficile* [31]. Several studies have observed the efficacy of different probiotic strains in the treatment of antibiotic/*C. difficile*-associated diarrhea. *L. acidophilus*, *L. rhamnosus* GG, *L. delbrueckii*, and *L. fermentum* are several bacteria that have been shown to decrease the occurrence of antibiotic-induced diarrhea [10]. *C. difficile*, a main concern with the usage of antibiotics pathogenesis, is the disruption of indigenous intestinal microbiome. Probiotics were shown in several studies to decrease *C. difficile* risks; those studies had several limitations such as the type of probiotic variation, the duration of use, and different dosages [35]. Therefore, *C. difficile* and probiotic relationship require more in-depth research.

There have been a variety of studies that observe and prove the health benefits and clinical effects of probiotics to GI abnormalities such as irritable bowel syndrome, gastric ulcer, and antibiotic-associated diarrhea and some cancers [8]. *Lactobacillus* and *Bifidobacteria* influence on resident microbiota can range from temporarily replacing missing parts or supplementing certain population, or by stimulating some of the resident microbiota. *Lactobacillus* species, which has been noted in several studies to provide beneficial effects when they are presented, is metabolically active and contains several properties that affect the whole intestinal microbiota biodiversity [4]. Prebiotics have been shown to suppress indigestion and diarrhea that were caused by pathogens [2].

Continuing, they can also aid in preventing the growth of harmful competitors, prevent the growth of exogenous microbes, and lower the substrate availability for pathogens [19]. *L. fermentum* ME-3 has been found able to suppress Gram-negative bacteria. Some probiotics have an antagonistic effect such as *L. paracasei* and *L. plantarum* with *Salmonella* (microaerobic), *L. plantarum* against *C. difficile* colitis (anaerobic), *L. paracasei* against *Helicobacter pylori*, and *B. lactis*/*B. longum* against *Shigella sonnei* and *E. coli*. Inflammatory bowel disease, which consists mainly of ulcerative colitis and Crohn's disease, has been shown related to intestinal flora dysbiosis through clinical and research studies. In an analysis of several studies, probiotics were determined to have a better outcome than non-probiotics therapy for maintenance therapy. However, they did not give benefit in inducing the remission of ulcerative colitis. This could be due to various methods used, different sample sizes, and controlled variables [34].

Prebiotics can also influence the composition of bacteria in human gut [9]. Several studies showed that when given supplements of fructan and inulin, there was an increased number of *Bifidobacteria* [19]. Other types of prebiotics that have been found to positively affect the gut microbe are arabinoxylan and inulin. These two have a modifying ability through affecting the makeup of and function ability of gut microbe [12]. *Bifidobacteria* and *Lactobacilli* selected fermentation of prebiotics have supported symbiotic gut microbiota through improving numbers of these commensals and decreasing the number of neutral

or pathogenic organisms. It is vital to know that these microbes' preference to coproduce certain fermentation products depend on the prebiotic structures and the bacterial communities [2]. Example of acidic fermentation products are lactate and short-chain fatty acid, butyrate acetate, and propionate [2]. These products can have benefits in the gut, for example, butyrate supports intestinal epithelium, and along with other SCFAs, they have benefits that are distal to the gut system.

4. Current recommendation for its use and types of availability

Before recommendations on probiotics are made, the following are needed to be taken into account: an individual's immunity, genetics, and diet [9]. The type of probiotics being suggested may differ based on goals and shelf life. World Health Organization (WHO) suggests that in order to provide health benefits, probiotics must be able to endure human digestion including gastric juices and bile and be capable of multiplying once they arrive in the GI tract [9]. The focus should be on the origin of the strain, its colonizing ability, and its safety and efficacy [4]. The amount varies based on the goal; however, it must be adequate enough to have colonization and effect [30]. The duration of the effect varies from probiotics. Studies have found that most effects only last as long as they are consumed [31].

There are an increasing number of probiotic products made available to consumers which include yogurt, other fermented milk and food products as well as various forms of dietary supplements [9]. Individual preferences can vary on the method of ingestion of probiotics. These are usually prepared using lactic acid bacteria of four general species, *Lactobacillus*, and *Bifidobacterium*. Probiotics are used in a variety of food sources not only in traditionally fermented food but are now being added to meat products, snacks, fruits, and juices [5]. Functional properties that lead to microorganisms in fermented foods have probiotics properties, antimicrobial properties, fibrolytic activity, and degradation of antinutritive compounds which may be essential when looking into the selection of a starter culture to be used in the makeup of functional foods [5].

In terms of prebiotics, foods such as artichoke, asparagus, garlic, and wheat have a variety of compound types that have been looked at for prebiotic attributes such as various length oligosaccharides and galacto-oligosaccharides/trans-oligosaccharides [2]. Monosaccharides, di-, and tri- may be used for prebiotics if they have host-indigestible bonds. Other examples of what have been used are sugar alcohol, cycle disaccharide difructose, and hydride II [2]. In order to have a beneficial effect from prebiotics, usually an individual will need 5 g or more to produce enough fermentation [1]. However, to avoid risk related to fermented food, a maximum limit of 100 mg/kg of histamine indicates safe level for consumption [6].

It is vital to recognize that there are no standard guidelines currently existing for oral administration, and the individual use of probiotic and prebiotics should be carefully monitored in order to determine potential adverse reaction [7]. More long-term well-controlled double-blinded studies are needed.

5. Summary

Gut microbial is essential for the balance of pathogens and the control of disease not only at the gastrointestinal tract but also distal to the tract as well. Metabolism and energy balance are major components of cardio-metabolic health [24]. Disease state has been determined to be one cause of an alteration to gut microbial that can affect the stated components. This was observed through different types of microbial environments in patients who are obese or diabetic. This change in gut microbial increases the disease state through the support of its pathogenesis.

Dietary supplements, including probiotics, could lower the risk of diseases such as CVD [17]. Probiotics have been known to cause a positive alteration in the gut microbial. They are often provided with prebiotics in fermented food and are termed synbiotic. Several studies have observed probiotics, its effect on gut microbial, and its relationship to cardiovascular diseases and risks. Probiotics may offer an alternative treatment for diabetes, obesity, hypercholesterolemia, hypertension, CKD, cardiomyopathy, atherosclerosis (**Table 1**). In order for a better understanding on how probiotics can lower the risks for diseases and treat, more studies need to be performed.

	Microorganism	**Results**	**Authors**
Obesity	***Lactobacillus rhamosus***	Mean weight loss in women was significantly higher than that in women in placebo group ($p = 0.02$)	Sanchez et al. [38]
Diabetes	***L. acidophilus*, *L. rhamnosus* and *B. bifidum***	Decline in blood glucose levels by 38% in T2DM subjects	Moroti et al. [39]
	***L. acidophilus* and *Bifidobacterium lactis* Bb12**	Significantly lowered fasting blood glucose hemoglobin A1c and malondialdehyde and increased erythrocyte superoxide dismutase and glutathione peroxidase activities and total antioxidant states	Ejtahed et al. [37]
Cholesterol	***L. acidophilus*** w. *B. longum*	Elevation of HDL cholesterol level by 0.3 mmol L-1 and reduction in the ratio of LDL/HDL cholesterol from 3.24 to 2.38	Kiessling et al. [42]
	L. reuteri	A significant reduction in LDL cholesterol 8.92%, total cholesterol 4.81%, non-HDL cholesterol 6.01%	Jones et al. [40]
	L. curvatus **and** *L. plantarum*	An increase of 21.1 and 15.6% in plasma apo A-V levels and LDL particles size	Ahn et al. [16]
Hypertension	***L. casei*** w/*Streptococcus thermophiles*	Systolic pressure lowered significantly ($p < 0.05$)	Kawase et al. [36]

Table 1. Main probiotic effect on cardiovascular disease risk-related states.

Each single strain of multiple strains must be observed individually. This is in order to directly compare the effectiveness of individual strain versus multi-strain [1]. Also, a synergistic effect in the bioactivity of probiotics could result in multi-strain, which can lead to a mutual inhibition by a component strain. This could possibly decrease probiotic efficacy [1]. While there are several probiotics that are available, only some have been shown to be effective and able to colonize [30].

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Chapter 4

Probiotic Applications in Autoimmune Diseases

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Additional information is available at the end of the chapter

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Abstract

Evidences from animal models and humans have implied the involvement of alterations in the gut microbiota in development of some autoimmune diseases. Dysbiosis observed in autoimmune diseases is associated with decreased bacteria function and diversity, impaired epithelial barrier function, inflammation, and decreased regulatory T cells in the gut mucosa. Studies suggest that probiotics influence systemic immune responses, ensure the homeostasis of the healthy microbiota in the intestinal mucosa, and could, therefore, be used as adjuvant therapy to treat immune-mediated diseases. The mechanisms proposed to achieve this include mucus secretion; antimicrobial peptide production; the maintenance of the function of the gastrointestinal-epithelial barrier, ensuring adequate interactions between the gut microbiota and the mucosal immune cells; and, finally, helping the activation of host immune system in response to pathobionts. Here, we described several reports concerning probiotic applications in several animal models of autoimmune diseases and data of the main clinical trials concerning the applicability of probiotics in type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus.

Keywords: dysbiosis, barrier disruption, inflammation, autoimmunity, probiotics

1. Introduction

Thousands of years ago, Hippocrates, father of medicine, coined the concept that food would serve as medicine and postulated, "Let food be thy medicine, and let medicine be thy food." Nowadays, the concept of food as a medicine appeared as functional foods, referring to any foods or ingredients with nutritional value and that promote a health benefit to the host [1]. Probiotics, prebiotics, and synbiotics are the most popular ingredients used as functional foods and dietary supplements [2].

According to the World Health Organization (2002) and the International Scientific Association for Probiotics and Prebiotics (2013), probiotics is defined as "a live organism, which provides a benefit to the host when provided in adequate quantities" [2–4]. Most commonly used probiotic includes lactic acid-producing bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species. Non-lactic acid-producing bacteria, such as *Bacillus* and *Propionibacterium*, species and nonpathogenic yeasts, including *Saccharomyces boulfecesardii*, non-spore-forming and non-flagellated rod or coccobacilli, and some helminths, such as *Trichuris suis ova*, could also been used as probiotics [5, 6]. Some of these strains were chosen based on origin, in vitro adherence to intestinal cells, and survival during passage through the gastrointestinal tract [5].

2. Intestinal dysbiosis in autoimmune diseases

Evidence from animal models has implied the involvement of intestinal dysbiosis in development of some autoimmune diseases [24–26]. Dysbiosis observed in autoimmune diseases is associated with decreased bacteria function and diversity, impaired epithelial barrier function, inflammation, and decreased regulatory T cells (Treg cells) in the gut mucosa [7, 8]. The hypotheses proposed to link dysbiosis with autoimmune diseases include molecular mimicry, bystander T cell activation, and the amplification of autoimmunity by pro-inflammatory cytokines, which is elicited by dysbiotic gut microbiota [9]. In 2016, Lerner and colleagues, from Institute Wendelsheim, in Germany, proposed the posttranslational modification of luminal proteins, promoted by enzymes from altered microbiota, which modify substrates in a different way than performed under homeostatic conditions. The defective posttranslational modification of luminal proteins could generate neo-epitopes that may become immunogenic and induce systemic autoimmunity and trigger autoimmune diseases [9].

Here, we described several reports concerning probiotic applications in several animal models of autoimmune diseases and data of the main clinical trials concerning the applicability of probiotics in type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus.

3. Probiotics in autoimmune diseases

Studies suggest that probiotics influence systemic immune responses, ensure the homeostasis of the healthy microbiota in the intestinal mucosa, and could, therefore, be used as adjuvant therapy to treat immune-mediated diseases [4]. The mechanisms proposed to achieve this include mucus secretion, antimicrobial peptide production, the maintenance of the function of the gastrointestinal-epithelial barrier, decreasing oxidative stress, ensuring adequate interactions between the gut microbiota and the mucosal immune cells, and, finally, helping the activation of host immune system in response to pathobionts [10].

3.1. Type 1 diabetes

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by autoimmune reactions against the insulin-secreting pancreatic β-cells, resulting in exogenous insulin dependence

to control blood glucose levels [11]. The etiopathogenesis may involve the interaction of predisposing human leucocyte antigens (HLA) alleles and environmental factors, such as viral infections, vitamin deficiencies, and disruption of the gut microbiota [12]. According to the International Diabetes Federation, more than 96,000 children and adolescents under 15 years will be diagnosed with T1D annually worldwide, and this number is estimated to be more than 132,600 when the age range extends to 20 years [13].

The role of the gut microbiota in T1D etiology has been the subject of research over the last decade to clarify its role in disease development and determine preventive approaches, such as diet manipulation and probiotic administration [12]. Several researches have been carried out to verify whether the administration of probiotics may improve the prognosis of diabetes through modulation of gut microbiota. Probiotics have been identified as effective adjuvants in insulin resistance therapies [14–16]. This health claims apparently stem from the ability of probiotics to secrete antimicrobial substances, competing with other pathogens, strengthening the intestinal barrier, and modulating the immune system [17].

3.1.1. Probiotics in animal models of autoimmune diabetes

The intestinal microbiota might modulate the autoimmune T1D pathogenesis via two mechanisms, recently proposed by Knip and Honkanen [18], from the University of Helsinki, in Finland. In the first phase, an impaired tolerance process in infancy leads to a susceptibility to develop autoimmune diseases, such as T1D, and may result in appearance of autoreactive T cells and autoantibodies. At the second phase, the intestinal dysbiosis predisposes children with genetic susceptibility and positive autoantibodies to develop clinical disease [18].

The inflammasome signaling components are innate immune sensors that are highly influenced by the gut environment and play pivotal roles in maintaining intestinal immune homeostasis [19]. Previous studies suggested the involvement of the gastrointestinal tract in the pathogenesis of islet autoimmunity. Thus, the modulation of gut-associated lymphoid tissue may represent a means to affect the natural history of the disease. Oral administration of probiotics can modulate local and systemic immune responses [20].

The earliest study to evaluate the efficacy of probiotics in T1D was published in 2005. The study performed by Calcinaro and colleagues, in the University of Perugia, in Italy, investigated the effects of oral administration of the probiotic VSL#3 in nonobese diabetic (NOD) mice development. VSL#3 was administered to female NOD mice three times a week starting from 4 weeks of age. Early oral administration of VSL#3 prevented diabetes development in NOD mice. Protected mice showed reduced insulitis and a reduced β-cell destruction. Prevention was associated with an increased production of interleukin (IL)-10 from Peyer's patches and the spleen and with increased IL-10 expression in the pancreas, where IL-10-positive islet-infiltrating mononuclear cells were detected. The protective effect of VSL#3 was transferable to irradiated mice receiving diabetogenic cells and splenocytes from VSL#3-treated mice. Oral VSL#3 administration prevents autoimmune diabetes and induces immunomodulation by a reduction in insulitis. These data provide a sound rationale for future clinical trials of the primary prevention of T1D by oral VSL#3 administration [21].

Eleven years later, Kim and colleagues evaluated the effects of *Bifidobacterium lactis* HY8101 on insulin resistance induced by tumor necrosis factor-alpha (TNF-α) in the skeletal muscle

cell from L6 rat. The treatment using HY8101 improved the insulin-stimulated glucose uptake and translocation of GLUT4 via the insulin signaling pathways AKT and IRS-1(Tyr) in TNF-treated L6 cells. HY8101 increased the mRNA levels of GLUT4 and several insulin sensitivity-related genes in TNF-α-treated L6 cells. HY8101 improved diabetes-induced plasma total cholesterol and triglyceride levels and increased the muscle glycogen content. *Bifidobacterium lactis* HY8101 can be used to moderate glucose metabolism, lipid metabolism, and insulin sensitivity in mice and in cells. *Bifidobacterium lactis* HY8101 might have potential as a probiotic candidate for alleviating metabolic syndromes such as diabetes [22].

Another work, performed in Yale University, by Peng and colleagues, in 2014, demonstrated that the protection from T1D development observed in MyD88-deficient NOD mice (MyD88−/− NOD) could be transferred to wild-type NOD mice [23, 24]. The gut bacteria isolated from MyD88−/−NOD mice, administered over a 3-week period, altered the family composition of the gut microbiome, mainly increasing the *Lachnospiraceae* and Clostridiaceae members and decreasing *Lactobacillaceae* family members. The gut microbiota-transferred mice had a higher concentration of IgA and transforming growth factor-beta (TGF-β) in the lumen that was accompanied by an increase in CD8+CD103+ and CD8$\alpha\beta$ T cells in the lamina propria of the large intestine. The data obtained in this study suggest that gut bacterial composition can be altered after the neonatal period, affects the mucosal immune system, and might delay the onset of autoimmune diabetes. These results have important implications for the development of probiotic adjuvant treatment for T1D [24].

In 2015, Le and colleagues, from the National Institute for Food Control, by using C57BL/6 J mice with streptozotocin-induced diabetes, evaluated whether *Bifidobacterium* species induce the expression of proteins of the insulin signaling pathway and enhance adipocytokine gene expression. Oral administration of *Bifidobacterium* species significantly reduced blood glucose levels and increased the protein expressions of insulin receptor beta, insulin receptor substrate 1, protein kinase B (Akt/PKB), IκB kinase alpha (IKKα), and nuclear factor-kappaB inhibitor alpha (IκBα). *Bifidobacterium* species also induce the adiponectin gene expression and decrease in macrophage chemoattractant protein-1 (MCP-1) and IL-6 expression. In conclusion, the results from this work suggest that *Bifidobacterium* species may be the promising bacteria for treat diabetes [25].

A study performed in Diabetes Research Institute, in Milan, Italy, by Dolpady and colleagues, in 2016, reported that the oral administration of a *Lactobacillaceae*-enriched probiotics VSL#3, alone or in combination with retinoic acid, protects NOD mice from diabetes by suppressing inflammasome activation and IL-1β expression and by inducing the immunomodulatory indoleamine 2,3-dioxygenase (IDO) and IL-33 secretion. In addition, VSL#3-treated NOD mice showed modulation of the gut immunity by promoting differentiation of CD103+ tolerogenic dendritic cells and suppressing the differentiation of inflammatory Th1 and Th17 subsets in the gut mucosa [26].

Accumulating evidence supports that the intestinal microbiome is involved in T1D pathogenesis through the gut-pancreas axis. A recent study, performed in the University of British Columbia, in Canada, Brown and colleagues [27], aimed to determine whether the gut microbiota in the NOD mice played a role in T1D through the gut mucosa. To examine the effect of the intestinal

microbiota on T1D onset, scientists manipulated gut microbes by fecal transplantation between NOD and resistant NOD mice (NOR) and by oral antibiotic and probiotic treatment of NOD mice. The intestinal microbiota from NOD mice harbored more pathobionts and fewer beneficial microbes in comparison with NOR mice. Fecal transplantation of NOD microbes induced insulitis in NOR hosts, suggesting that the NOD microbiome is diabetogenic. Moreover, antibiotic exposure accelerated diabetes onset in NOD mice accompanied by increased Th1 and Th17 cells in the mucosal-associated lymphoid tissues. The diabetogenic microbiome was characterized by a metagenome altered in several metabolic gene clusters. Furthermore, diabetes susceptibility correlated with reduced fecal short chain fatty acids. In an attempt to correct the diabetogenic microbiome, researchers administered VLS#3 probiotic to NOD mice and found that VSL#3 colonized the intestine poorly and did not delay diabetes onset. Authors concluded that NOD mice harbor gut microbes that induce diabetes and that their diabetogenic microbiome can be amplified early in life through antibiotic exposure. Protective microbes like VSL#3 are insufficient to overcome the effects of a diabetogenic microbiome [27].

Another recent work, performed in Jiangnan University, in China, Jia and colleagues [28], investigated whether administration of probiotic *Clostridium butyricum* CGMCC0313.1 (CB0313.1) could induce Treg cells in pancreas, and consequently inhibit the diabetes onset in NOD mice. CB0313.1 supplementation was delivered daily to female NOD mice from 3 to 45 weeks of age. Researchers observed that probiotic administration suppressed the insulitis, delayed the disease onset, and improved the glucose metabolism. These beneficial effects could involve the migration of intestinal Treg cells to the pancreatic lymph nodes and changes in the Th1/Th2/Th17 balance, favoring an anti-inflammatory milieu in the gut and pancreas. Additionally, probiotic supplementation increased the Firmicutes/*Bacteroidetes* ratio, *Clostridium* species, and butyrate-producing bacteria in the gut [28].

3.1.2. Probiotic applications in T1D patients

Probiotic supplementation has been hypothesized to affect innate and adaptive immune responses to environmental antigens by supporting healthy gut microbiota and could therefore be used to prevent the onset of T1D-associated islet autoimmunity and treat the established disease [29].

In humans, a TEDDY study group, published in JAMA Pediatrics in 2016, evaluated the association between probiotic supplementation and islet autoimmunity in children with genetic risk for T1D, during their first year of life. This multicenter prospective cohort study (United States, Finland, Germany, and Sweden) investigated 7473 children ranging from 4 to 10 years old. Early probiotic administration (0–27 days of life) was correlated with a decreased risk of islet autoimmunity when compared with the group that received probiotics after 27 days of life or no supplementation. This study concludes that early probiotic supplementation could decrease the risk of islet autoimmune reactions in children with high-genetic-risk alleles for T1D [30].

A current clinical trial, performed by Medical University of Warsaw, in Poland, involves the evaluation of the effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 on β-cell function in children with newly diagnosed T1D. The double-blind, randomized, placebo-controlled clinical trial included 96 children aged 8 to 17 years old. During 1 year, patients

received *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 at a dose of 10^9 colony-forming units or an identically appearing placebo, orally, daily, for 6 months. The follow-up will be for 12 months. The primary outcome measures will be the area under the curve of the C-peptide levels during 2 h response to a mixed meal [31].

The *Lactobacillus* and *Bifidobacterium* are the major bacteria genera that make up the colon microbiota in humans and help in the intestinal microbial homeostasis, inhibit growth of pathobionts, improve the gut mucosal barrier, and modulate local and systemic immune responses. Intestinal dysbiosis may influence the immune system by increasing gut permeability, intestinal inflammation, and impaired oral tolerance in T1D patients. Beneficial effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 on β-cell function would create a rationale for its routine use in patients with newly diagnosed T1D [31]. Taken together, the studies imply that bacteriotherapy may potentially be used as a tool to modulate the immune system for preventing islet autoimmunity [31, 32].

3.2. Multiple sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory, autoimmune disease that affects the central nervous system (CNS) and is characterized by immune reactions against myelin proteins and gangliosides. Susceptible HLA alleles and environmental factors, such as virus infection, a hypercaloric diet, vitamin D deficiency, and intestinal dysbiosis, have been implicated in triggering MS [33]. MS promotes disability in young adults and affects twice more women than men. According to the Multiple Sclerosis International Federation and World Health Organization, the prevalence of MS increased from 2.1 million in 2008 to 2.3 million in 2013 [34].

Studies have shown that gut microbiota can affect the development of MS, and these works implicated intestinal dysbiosis as one of the possible causes of extraintestinal disease development [35]. The colonization of germ-free mice with segmented filamentous bacteria promotes an increase in the number of Th17 cells in the lamina propria and CNS, worsening disease severity in experimental autoimmune encephalomyelitis (EAE), a MS animal model [36]. Likewise, the colonization of the same mice with *Bacteroides fragilis* and polysaccharide A (PSA), which induces Foxp3+ Treg cell differentiation, decreases symptoms in EAE mice [37].

3.2.1. Probiotics in experimental autoimmune encephalomyelitis

Several studies in experimental autoimmune encephalomyelitis (EAE) mice reported the immunomodulatory functions of probiotic administration. Treatment with *Lactobacillus* species, *Pediococcus acidolactici*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, and *Bacteroides fragilis* decreased CNS inflammation through the induction of Treg cells in the gastrointestinal mucosa, IL-10 and TGF-β secretion, and decreased expansion of Th1 and Th17 inflammatory subsets [37–40].

In previous studies, performed in National Institute for Public Health and the Environment, in the Netherlands, in 2008, Ezendam and colleagues evaluated the effect of the probiotic *Bifidobacterium animalis* on Th1- and Th2-mediated immune responses, including a rat EAE model. *Bifidobacterium animalis* administration started when the rats were 2 weeks old and EAE were induced when the animals were 6–7 weeks old. *Bifidobacterium animalis* significantly

reduced the duration of clinical symptoms by almost 2 days in males and improved the body weight gain during the experimental period compared with the control group [41]. In the same year, Maassen and colleagues presented data showing that strain-specific differences on the effect of commercially available probiotic depend on physiological use (normal route, dose, growth phase, specific strain, or substrain/species) and overwhelm (high dose) or circumvent natural immune processing [42].

Two years later, Lavasani and colleagues, from Lund University, in Sweden, evaluated the effect of five daily-administered *Lactobacillus* strains in inhibiting disease onset in EAE mice. The *Lactobacillus paracasei* DSM 13434 and *Lactobacillus plantarum* DSM 15312 and DSM 15313 diminished autoreactive T cell responses and inflammation in the CNS. *Lactobacillus paracasei* and *Lactobacillus plantarum* DSM 15312 induce Treg cells in mesenteric lymph nodes and TGF-β secretion. *Lactobacillus plantarum* DSM 15313 induces increase in the IL-27 serum concentrations. The isolated *Lactobacillus* strains failed to be therapeutic in EAE mice. On the other hand, the combination of three strains inhibited the disease progression and reversed the clinical and histological signs of EAE, probably by suppressing inflammatory Th1 and Th17 pathways and inducing regulatory mechanisms [43].

In 2010, Kobayashi and colleagues, from Yakult Central Institute for Microbiological Research, in Japan, evaluated the safety of two probiotic bacterial strains, *Lactobacillus casei* strain Shirota (LcS) and *Bifidobacterium breve* strain Yakult (BbY), that were orally administered to EAE Lewis rats. EAE was induced with a homogenate of guinea pig spinal cord as the sensitizing antigen, and LcS was orally administered from 1 week before this sensitization until the end of the experiment. The oral administration of LcS tended to suppress the development of neurological symptoms. Differences in neurological symptoms between the control group and the administration groups did not reach statistical significance and support the notion that neither LcS nor BbY exacerbates EAE [44].

Two years later, Kobayashi and colleagues investigated the safety use of *Lactobacillus casei* strain Shirota (LcS) in prevention of EAE in a relapse and remission models. LcS was administered 1 week prior antigen sensitization until the end of the experiments. Probiotics did not exacerbate neurological symptoms or histopathological changes of the spinal cord in either model. LcS administration transiently induces IL-17 production by antigen-stimulated lymphocytes 7 days after sensitization. Increased production of IL-10 and an increase in the percentages of CD4+CD25+ Treg cells were observed. Strong expression of IL-17 mRNA was detected in the spinal cord of mice that displayed severe neurological symptoms on day 12, but this expression was not enhanced by LcS administration [45].

In 2013, Kwon and colleagues, from School of Life Sciences and Immune Synapse Research Center, in Republic of Korea, evaluated the prophylactic and therapeutic actions of a mixture of five probiotics (IRT5) in EAE mice. IRT5 includes *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Bifidobacterium bifidum*, and *Streptococcus thermophilus*. IRT5 prior treatment, before EAE induction, abrogated the disease development and delayed the EAE onset. Furthermore, the inflammatory subset Th1 and Th17 polarization was suppressed by the administration of IRT5 probiotic. These actions were due probably by induction of CD4+Foxp3+ Treg cells and IL-10 secretion at sites of inflammation and peripheral lymph nodes [46].

Three years later, Abdurasulova and coworkers, from Institute of Experimental Medicine, in St. Petersburg, Russian Federation, evaluated the effect of probiotic *Enterococcus faecium* strain L-3 that was studied in EAE rats. Glatiramer acetate (GA) was used as control drug. *Enterococcus faecium* strain L-3 and GA were able to reduce the severity of EAE. Both approaches prolonged the inductive phase of EAE and reduced the disease duration. Study of the phenotypes of immune cells in the blood revealed the differences in immunoregulatory pathways that mediate the protective action of probiotic or GA treatment of EAE. The presence of pronounced protective and immunomodulating effects of the probiotic *Enterococcus faecium* strain L-3 opens an opportunity of its application for the adjuvant treatment of MS [47].

The Goudarzvand group [48], from School of Medicine, in Karaj, Iran, investigated the effect of *Lactobacillus plantarum* (LP) and *Bifidobacterium* B94 (BB94) on acquisition phase of spatial memory in the local demyelination of rats' hippocampus. Thirty-two male Wistar rats were divided into control, damage group and treatment group. After the induction of demyelination, probiotics were administered by gavage for 28 days. Findings demonstrated that probiotics have no significant effect on swimming speed compared with lesion and saline groups. According to some studies, probiotics have a positive impact on improving the performance of spatial memory and learning, although this current study could not indicate finality of this assumption [48].

A recent study, performed by Secher and colleagues [49], from the University of Toulouse, in France, evaluated the effects of the probiotic *Escherichia coli* strain Nissle 1917 (ECN) in EAE model. The daily oral administration of ECN significantly decreased the disease severity induced by myelin oligodendrocyte glycoprotein (MOG) peptide mice immunization. The therapeutic effects could be explained by the increase in the IL-10 anti-inflammatory cytokine and reduction in inflammatory cytokines in the CNS and in the periphery. They also observed a decreased frequency of MOG-specific CD4+ T cells in the CNS, suggesting that ECN modulate the T cell homing from the lymph nodes to the CNS by affecting their activation and differentiation. In this study, authors showed that EAE trigger is associated with increased gut permeability [49].

Another recent study, performed in Immunology Research Center, in Mashhad, Iran, Salehipour and colleagues [50], evaluated the therapeutic effect of probiotic strains, *Lactobacillus plantarum* A7, *Bifidobacterium animalis* PTCC 1631, or both. Probiotics were administered orally for 22 days starting at same time with the induction of EAE in female C57BL/6 mice. Results showed that treatment with both strains caused a more significant delay in the time of disease onset and clinical score compared with strains used alone. Mononuclear cell infiltration into the CNS was significantly inhibited by the combinational approach. The treatment with both strains enhanced the population of CD4+CD25+Foxp3+ Treg cells in the lymph nodes and spleen. Additionally, *Lactobacillus plantarum* A7 and *Bifidobacterium animalis* ameliorated EAE condition by inhibiting IL-6 production, decreasing the release of IFN-γ, a Th1-type cytokine, and IL-17, a Th17 pro-inflammatory molecule, and increasing the secretion of IL-4, a Th2-type cytokine, and IL-10 and TGF-β, anti-inflammatory cytokines, in the lymph nodes and spleen. The treatment with *Bifidobacterium animalis* induced a downregulation of transcription factors T-bet and ROR-γt that generate Th1 and Th17 inflammatory subsets, in the brain and spleen, and promoted an upregulation of GATA3 and Foxp3, which contributes for the Th2 and Treg cell differentiation [50].

3.2.2. Probiotic applications in MS patients

Probiotic applications based on the hygiene hypothesis, such as administration of the eggs from nonpathogenic helminth *Trichuris suis ova* (TSO), have proven safe and effective in autoimmune inflammatory bowel disease. Based on this, Fleming and colleagues [6], from the University of Wisconsin, in the United States, evaluated the safety and effects of TSO administration in newly diagnosed, non-treated relapsing-remitting MS patients. Researchers conducted the phase 1 helminth-induced immunomodulatory therapy (HINT 1) study by enrollment of five MS patients that took orally 2500 TSO, every 2 weeks, for 3 months. The preliminary outcomes showed increase in the serum levels of IL-4 and IL-10 cytokines and decreased in the mean number of new gadolinium-enhancing magnetic resonance imaging (MRI) lesions. TSO was well tolerated in this first human study of the probiotic application in relapsing-remitting MS, and favorable trends were observed in exploratory MRI and immunological parameters [6].

Two years later, Rosche and colleagues, from the Department of Neurology and Experimental Neurology, in Berlin, Germany, evaluated the administration of 2500 *Trichuris suis ova* eggs orally, every 2 weeks, for 12 months, in relapsing-remitting MS patients. Fifty patients with relapsing-remitting MS with clinical activity, not undergoing any standard therapies, were enrolled. The safety, tolerability, and effect on disease activity and in vivo mechanisms of action of TSO in MS will be assessed by neurological, laboratory, and immunological exams and MRI throughout the 12-month treatment period and over a follow-up period of 6 months. No adverse effects were observed, and the *Trichuris suis ova* group was more effective than the placebo in preventing new T2 and gadolinium-positive lesions, quantified by MRI. Authors also expect the Th1 and Th17 pro-inflammatory responses polarize toward the anti-inflammatory Th2 response [51].

In a recent study, Kouchaki and colleagues [52], from School of Medicine from Kashan, in Islamic Republic of Iran, reported improved Expanded Disability Status Score (EDSS), insulin resistance, and a decrease in inflammatory markers in MS patients treated with probiotic supplementation containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, and *Bifidobacterium bifidum*. This randomized double-blind, placebo-controlled clinical trial analyzed probiotic intake for 12 weeks in 60 MS patients. Compared with the placebo group, probiotic administration improved EDSS, beck depression inventory, general health questionnaire, and depression anxiety and stress scale. Furthermore, changes in high-sensitivity C-reactive protein, plasma nitric oxide metabolites, and malondialdehyde in the probiotic group were significantly different from the changes in these parameters in the control group. In addition, the probiotic intake significantly decreased insulin levels and total high-density lipoprotein (HDL) cholesterol and significantly increased quantitative insulin sensitivity check index and HDL-cholesterol levels compared with the placebo [52].

Another recent randomized, double-blind, placebo-controlled clinical trial, performed in Islamic Republic of Iran, by Tamtaji and colleagues [53], evaluated the role of probiotic administration on gene expression associated to inflammatory, glucose, and lipid signaling pathways in MS patients. The study included 40 patients with MS. Participants were randomly assigned into two groups to receive either a probiotic capsule containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* (2×10^9 colony-forming units/g each) or placebo, for 12 weeks. Researchers observed that probiotic administration

downregulated gene expression of IL-8 and TNF-α mRNA in peripheral blood mononuclear cells of MS patients. On the other hand, probiotics did not affect the gene expression of IL-1, peroxisome proliferator-activated receptor gamma (PPAR-γ), or oxidized low-density lipoprotein receptor (LDLR) in peripheral blood mononuclear cells of MS patients [53].

3.3. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic inflammation of multiple joints, bone erosion, and cartilage destruction. Moreover, RA can affect internal organs such as the lungs, heart, and kidneys. Anti-cyclic citrullinated peptide and rheumatoid factor are the most important autoantibodies in RA and can be found before disease onset [54]. The disease is three times more common in women, and according to the World Health Organization, the worldwide prevalence, which is between 0.3 and 1%, ranks the disease among the most common autoimmune disorders. The triggering of RA involves the interaction of HLA genes and environmental factors, such as smoking and infections [55]. Among environmental factors, dysbiosis has been identified as a possible trigger factor for autoimmunity and RA development [56].

3.3.1. Probiotics in animal models of RA

Experiments in animal models suggest that gut microbiota influences local and systemic immunity and might trigger joint inflammation [57]. Studies in collagen-induced arthritic (CIA) mice showed that the administration of antibiotics exacerbates the disease and increases the level of IL-6, IFN-γ, and IL-17 pro-inflammatory cytokines. Further study showed differences in the gut microbiota composition between CIA-susceptible and CIA-resistant mice, with a prevalence of *Desulfovibrio*, *Prevotella*, *Parabacteroides*, *Odoribacter*, *Acetatifactor*, *Blautia*, *Coprococcus*, and *Ruminococcus* genera in arthritic mice, in addition to increased levels of serum IL-17 and CD4 Th17 cells in the spleen [58].

The study performed by Abhari and colleagues [59], in Shiraz University, in Iran, investigated the possible role of probiotic *Bacillus coagulans* and prebiotic inulin on the downregulation of immune responses and the progression of RA, by using rat models of the disease. The spore-forming probiotic strain *Bacillus coagulans* has an anti-inflammatory and immunomodulatory effects in animals and humans. The treatment with the probiotic and prebiotic significantly inhibits serum amyloid A in arthritic rats, and a significant decrease in the secretion of the pro-inflammatory TNF-α was detected [59].

Another work, performed in the Department of Probiotics Immunology, Sapporo University, in Japan, Yamashita and colleagues [60], evaluated the effect of the oral administration of *Lactobacillus helveticus* SBT2171 on CIA development and on the regulation of antigen-specific antibody production and inflammatory immune cells, implicated in the RA development. Probiotic administration promotes decrease in joint swelling, body weight loss, and the serum level of bovine type II collagen (CII)-specific antibodies in the CIA mouse model. In addition, the intraperitoneal inoculation of *Lactobacillus helveticus* SBT2171 also decreased the arthritis incidence, joint damage, and serum concentrations of IL-6. Furthermore, the numbers of total immune cells, total B cells, germinal center B cells, and CD4+ T cells in the draining lymph nodes were decreased following intraperitoneal inoculation of *Lactobacillus helveticus* SBT2171. Findings of this study

demonstrated the ability of *Lactobacillus helveticus* SBT2171 to downregulate the abundance of immune cells and the subsequent production of CII-specific antibodies and IL-6, thereby suppressing the CIA symptoms, indicating its potential for use in the prevention of RA [60].

Lactobacillus helveticus SBT2171 (LH2171) is a lactic acid bacterium with high protease activity and used in starter cultures in the manufacture of cheese. Scientists have demonstrated that LH2171 inhibited the proliferation of lipopolysaccharide (LPS)-stimulated mouse T and B cells and the human lymphoma cell lines, Jurkat and BJAB. The findings of this study suggest that LH2171 inhibits the proliferation of lymphocytes through the suppression of the JNK signaling pathway and exerts an immunosuppressive effect in vivo, reinforcing their use in treatment of immune-mediated diseases [61].

Intestinal dysbiosis has been previously identified in patients with RA, and the administration of certain probiotics showed an improvement in RA. Study from Gohil and colleagues [62], from the Institute of Pharmaceutical Education and Research, in Gujarat, India, was designed to find out the antiarthritic activity of cell wall content of *Lactobacillus plantarum* in complete Freund's adjuvant (CFA)-induced arthritis in rats. The change in body weight, paw volume and arthritic index, joint stiffness, gait test, mobility test, erythrocyte sedimentation rate, serum C-reactive protein level, serum rheumatoid factor, and serum TNF-α was measured on day 21. Cell wall content of *Lactobacillus plantarum*-treated animals showed improvement in all the parameters as compared to that in CFA-treated animals and exert antiarthritic activity [62].

3.3.2. Probiotic applications in RA patients

Some performed studies evaluating the effect of probiotics as an adjuvant therapy for RA treatment have shown no significant results, and some of these conducted studies have smaller number of patients and a short period of evaluation [63, 64].

The earliest study to evaluate the efficacy of probiotics in RA was performed in Rheumatism Foundation Hospital, in Finland, and was published in 2003. In a pilot study, Hatakka and colleagues evaluated 25 non-treated RA patients that were randomized to receive either two capsules of a *Lactobacillus rhamnosus* or placebo, twice daily for a year. Overall, no statistically significant differences were seen between the case and the placebo. Both groups had a decline in tender and swollen joints, and the physician global scores improved in the probiotic group. Mean erythrocyte sedimentation rates and C-reactive protein levels remained normal in both groups. The serum concentrations of IL-1β increase in patients treated with *Lactobacillus* species; however, this increase was not associated with any detectable change in disease status. Fecal sampling showed an increase in the presence of *Lactobacillus rhamnosus* in the probiotic group at 1 year. Based on these results, researchers concluded that *Lactobacillus rhamnosus* preparation did not alter RA activity. However, study cohort was small, and enrolled patients have low disease activity [65].

A double-blind, placebo-controlled clinical trial, performed in the University of Western Ontario, Canada, by Pineda and colleagues [63], evaluated the effect of the oral administration of *Lactobacillus rhamnosus* and *Lactobacillus reuteri* for 3 months to 29 RA patients. Fifteen patients were randomized to the probiotic group and 14 to placebo. Alterations in cytokines favored placebo over probiotic group. There was a significant improvement in the Health Assessment Questionnaire score in the probiotic group. Although researchers did not detect

clinical improvement, measured by the American College of Rheumatology criteria, authors reported functional improvement within the probiotic supplementation group compared with the placebo [63].

Another randomized, double-blind placebo-controlled trial, performed in Tabriz University of Medical Sciences, in Iran, by Vaghef-Mehrabany and colleagues [64], investigated the role of *Lactobacillus casei* 01 intake in 46 RA patients for 8 weeks. This clinical trial showed improvement in disease activity score, increased levels of serum IL-10, and decreased levels of pro-inflammatory TNF-α, IL-6, and IL-12 cytokines in treated patients. In this study, scientists concluded that supplementation improved the disease activity and inflammatory status in RA patients [64].

Another clinical trial, with the same study design, performed by Zamani and colleagues [66], in Kashan University of Medical Sciences, Iran, evaluated the effect of probiotic administration on clinical and metabolic parameters in RA patients. Sixty patients aged 25–70 years were enrolled into two groups to receive either probiotic or placebo. Probiotic group received a daily capsule containing three strains: *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*, for 8 weeks. After intervention, probiotic administration improved Disease Activity Score of 28 joints (DAS-28). In addition, a significant decrease in serum insulin levels, homeostatic model assessment-B cell function (HOMA-B), and serum high-sensitivity C-reactive protein concentration was also observed in the probiotic group [66].

3.4. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune and heterogeneous disease characterized by damage to the skin, kidneys, lungs, joints, heart, and brain [67]. The disease affects mainly females, and its worldwide prevalence varies from 30 to 60 per 100,000 in the United Kingdom and the United States [68]. SLE pathogenesis may involve genetic and environmental factors, such as viral infections, defective apoptosis, elevated oxidative stress, and solar exposure to ultraviolet-B waves. Regarding immune response, it is known that autoantibodies bind mainly with nuclear and cytoplasmic antigens [69]. Moreover, increased evidence has emerged in a recent year that suggests the role of intestinal dysbiosis in SLE development [70].

3.4.1. Probiotics in animal models of SLE

In female lupus-prone mice, Zhang and colleagues [71] reported a decrease in the relative abundance of *Lactobacillus* species and an increase in *Lachnospiraceae* members when compared with controls. Early disease onset and severe symptoms correlated with increased *Lachnospiraceae* reads in female lupus-prone mice. Additionally, the number of Clostridiaceae and *Lachnospiraceae* reads increased at specific time points during disease progression [71]. Another study reported that dietary intervention, such as caloric restriction, in NZB/WF1 mice promoted changes in the intestinal microbiota and delayed disease progression in this animal model [72].

In a lupus-like animal model, the administration of retinoic acid restored *Lactobacillus* species and improved lupus symptoms, suggesting the use of these species as a probiotic to diminish

inflammation in SLE patients [71]. Some *Lactobacillus* species have been demonstrated to have immunomodulatory properties in the host gut mucosa, such as inhibiting neutrophil extracellular trap formation, improving antioxidant status, and increasing the expression of adhesion molecules in the gut [73, 74].

In a recent study, performed by Tzang and colleagues [75], in Chung Shan Medical University, in Taiwan, scientists investigated the effects of oral administration of *Lactobacillus paracasei* GMNL-32, *Lactobacillus reuteri* GMNL-89, and *Lactobacillus reuteri* GMNL-263 in NZB/W F1 mice. When researchers evaluated the administration of the three probiotic strains, they observed a significant decrease in IL-6 and TNF-α serum concentrations and increase in antioxidant activity in serum and liver samples (higher glutathione GSH and 1,1-diphenyl-2-picrylhydrazyl levels and lower malondialdehyde levels). Additionally, the supplementation with *Lactobacillus reuteri* GMNL-263 significantly increased the differentiation of CD4+CD25+FoxP3+ Treg cells in NZB/W F1 mice, suggesting that these strains could be used as adjuvant treatment of SLE patients [75]. Another investigation from the same group demonstrated that supplementation with these three probiotic strains ameliorates hepatic apoptosis, matrix metalloproteinase-9 activity, C-reactive protein, and inducible nitric oxide synthase expressions. In addition, probiotics decrease the gene expression of hepatic IL-1β, IL-6 and TNF-α proteins, by suppressing the mitogen-activated protein kinase and NF-κB signaling pathways [76].

Although some studies in SLE animal models showed promising results using probiotic supplementation, currently, there are no clinical trials reported at clinicaltrials.gov investigating the role of probiotics as an adjuvant therapy in the treatment of SLE patients.

4. Conclusions

Evidences associate intestinal dysbiosis with autoimmune disease pathogenesis. Impaired gut microbiota function and diversity could represent a trigger site of autoimmunity by neoantigen generation under dysbiotic conditions. Emerging findings point to the use of probiotics as a preventive functional food and as adjuvant treatment of autoimmune diseases. However, further clinical trials, with large cohorts, to evaluate the security and efficacy of the probiotic administration in patients with autoimmune diseases are needed.

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Conflict of interest

The author reports no conflict of interest.

Appendices and nomenclature

Treg	T regulatory cells
T1D	Type 1 diabetes
NOD mice	Nonobese diabetic mice
IL	Interleukin
TNF-α	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor-beta
MCP-1	Macrophage chemoattractant protein-1
IDO	Indoleamine 2,3-dioxygenase
NOR mice	Resistant NOD mice
TEDDY	The Environmental Determinants of Diabetes in the Young
MS	Multiple sclerosis
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
PSA	Polysaccharide A
GA	Glatiramer acetate
MOG	Myelin oligodendrocyte glycoprotein
TSO	*Trichuris suis ova*
MRI	Magnetic resonance imaging
EDSS	Expanded Disability Status Score
HDL	High-density lipoproteins
RA	Rheumatoid arthritis
CIA mice	Collagen-induced arthritic mice
LPS	Lipopolysaccharide
CII	Type II collagen-specific antibodies
NZB	New Zealand black mice

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Chapter 5

The Role of Probiotics in Acne and Rosacea

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Abstract

Through basic science as well as animal and human clinical trials, the evidence is growing for the use of probiotics in the treatment of acne. Acne formation is dependent upon several processes, including follicular hyperkeratinization, excess sebum production, *Propionibacterium acnes* colonization and an inflammatory cascade. The antimicrobial properties of probiotics as well as the modification of the skin microbiome may decrease levels of *P. acnes* on the skin. Additionally, successful acne outcomes are influenced by compliance with topical regimens, which can commonly cause skin barrier disruption, leading to dryness and irritation. Consequently, calming inflammation as well as maintaining skin hydration and barrier repair is of primary importance when treating acne. In this chapter, we discuss how probiotics affect several factors in the pathophysiology of acne development and can improve the treatment outcomes.

Keywords: acne, probiotics, pathogenesis, inflammation, therapy

1. Introduction

Acne is an inflammatory disorder involving the pilosebaceous unit. A multifactorial cascade including excess sebum production, follicular hyperkeratinization, and bacterial overgrowth conspire to incite an inflammatory response. Acne therapies have focused on modulating this inflammatory response as well as targeting components of this cascade. Probiotics is an emerging area of research that continues to gain momentum for the treatment of acne. A probiotic is defined as a "live microorganism which, when administered in adequate amounts, confers a health benefit on the host" [1]. Both oral and topical preparations of probiotics have shown promise in the treatment of acne.

2. The gut-brain-skin axis

The field of dermatology continues to investigate the interconnected relationships between the skin and other systems of the body. The unifying theory of the gut-brain-skin axis outlines the relationship between the skin, gastrointestinal (GI) system, and mental health. Notably, the role of the gastrointestinal (GI) system is an area of particular interest as it relates to inflammatory skin conditions. The gut microbiome reacts to various stimuli resulting in systemic responses. By altering the GI microbiome, it is possible to decrease systemic inflammation, and these results can improve the severity of inflammatory skin diseases such as acne.

2.1. History of the gut-brain-skin axis

The gut-brain-skin axis originated in 1930 when John H. Stokes and Donald M. Pillsbury reported their clinical observations and colleagues' studies. They linked emotional states with gastrointestinal (GI) disorders through various mechanisms, including diet and neuronal responses. Stokes and Pillsbury reported cases of individuals with colitis who also suffered from urticaria and dermatographism. They purported that alterations of microflora increase gut permeability and lead to systemic inflammation. Higher levels of systemic inflammation result in altered cutaneous physiology. This association was further supported by the observation that hypochlorhydria is associated with multiple dermatologic conditions such as rosacea, eczema, pruritus, psoriasis, dermatitis herpetiformis, neurodermatitis, and acne [2].

Similarly, other case studies support the connection between gut physiology and cutaneous and psychological pathology. A psychopathic institute administered *Bacillus acidophilus* to their patients and recorded improvement in their mental distress, gastrointestinal disturbances, and skin eruptions [3]. A 1916 study showed patients with acne exhibiting alterations in their intestinal permeability [4]. In 1937, novel therapies for acne included acidophilus cultures, which acted as "intestinal flora changers" and improved pustular acne [5].

Stokes and Pillsbury's early concept of the gut being linked with the brain and skin started with anecdotal evidence and preliminary studies. They discovered that alteration of gastric acid levels and fluctuation of the gut microflora could have further effects beyond the GI system. From this evidence, they even proposed a treatment of Bacillus and cod liver oil, which is similar to present day probiotics and omega-3 fatty acids, to restore homeostasis in the gut. This early research has led to larger animal and human studies on the GI system, its microbiome, and its relation to the skin.

2.2. Theory of the gut-brain-skin axis

The negative impact of acne and other cutaneous diseases on the quality of life has been well-documented [6–10], and can cause long-lasting personality changes [10]. It has been reported that 8.8% of patients with acne exhibit depression [11]. Apart from psychological distress, acne patients often suffer from gastrointestinal disturbances at higher rates compared to the normal population [12]. The gastrointestinal tract houses the largest population of commensal bacteria, and in this reservoir of bacteria lies the central theme to the gut-brain-skin axis.

Hypochlorhydria and small intestinal bacterial overgrowth (SIBO) are two conditions that demonstrate an association with cutaneous pathology and mental health. Alterations in gastric acid secretion, such as hypochlorhydria, increase the risk for SIBO. While increased rates of SIBO have long been associated with psychological disorders of anxiety, depression, and fibromyalgia, more recent evidence has demonstrated increased rates of SIBO in rosacea patients, as well, with one study reporting an SIBO rate of 50% in rosacea patients. Those patients were then treated for SIBO with the antimicrobial rifaximin, and most had significant improvement or clearance of their rosacea [13, 14]. Additionally, an Australian study used the probiotic *Lactobacillus casei* to successfully reduce SIBO [15]. The authors not only noted improvement in the GI symptoms but also in cutaneous and psychological symptoms [13, 16, 17].

Intestinal permeability has also been linked to cutaneous pathology. As far back at 1916, acne patients have shown reactivity to stool-isolated bacteria using a serum complement test [4]. Later, Juhlin and Michaelsson tested *Escherichia coli* polysaccharide endotoxin in the blood samples of acne patients. The patients with severe acne had reactivity while the control had none [18]. A second study of human subjects with IBS identified increased levels of *E. coli* lipopolysaccharide (LPS)-induced cytokines in patients with IBS compared to controls [19]. Increased intestinal permeability and heightened immune responses has also been associated with chronic constipation [20]. Several studies have noted a higher prevalence of constipation in those with acne vulgaris [12, 21]. These correlations suggest increased intestinal permeability and consequently higher levels of circulating endotoxin may contribute to acne formation [21].

2.3. Impact of the gut microbiome

The gut microbiome is dynamic; it changes with the stress on its environment and reacts to feedback from other systems. As stated previously, changes in the microbiome may influence levels of systemic inflammation. Acne is an inflammatory disorder that has demonstrated its response to systemic inflammation and oxidative stress. A Russian study found that not only is the intestinal microflora of acne patients altered, but therapy for the GI disruption reduced the duration of acne treatment [22].

The gut's commensal bacteria can induce immune responses that ultimately reach T cells in the skin. Probiotics interact with the gastrointestinal mucosal immune system, altering levels of inflammatory cytokines in the blood [23]. For instance, the levels of gamma-aminobutyric acid (GABA) are modified by intestinal bacteria [24, 25]. Microbial-fermented food enriched with GABA has been shown to improve atopic dermatitis in mice through a Th-1 mediated-immune response [26].

Major histocompatibility cell (MHC) class II complexes are found on antigen presenting cells, such as dendritic cells and macrophages, and interact with immune-regulating T cells. Gastrointestinal bacteria, including strains of probiotics, have been shown to bind to the MHC II complex and modify their expressions [27]. A study that administered *Lactobacillus paracasei* NCC2461 (ST11) to mice found the probiotic to induce T regulatory cells and inhibited CD4+ T-cell proliferation, while increasing the secretions of anti-inflammatory cytokines, specifically IL-2, IL-10, and TGF-β [28]. Other strains of lactic acid bacteria continue to show that they induce T cell communication, decrease inflammatory response, and regulate antigen

presenting cells [29, 30]. The anti-inflammatory cytokines affect the differentiation of keratinocytes, while TGF-β has a considerable role in enhancement of the skin barrier [28, 31]. These findings were supported by a second study. Mice that were treated with *Lactobacillus casei* recruited T regulatory cells to inflamed skin and released higher levels of the anti-inflammatory cytokine IL-10 [32].

The composition of the gut microbiome can inhibit or promote the release of substance P in both the skin and intestinal tract [33, 34]. When a specific strain of *Lactobacillus paracasei* ST11 was orally administered, secretions of substance P decreased. Lower systemic levels of substance P enhanced skin barrier function and decreased local skin inflammation [35]. Inhibition of substance P directly affects acne pathogenesis, as substance P increases sebum production [36].

The interconnected relationship described by the gut-brain-skin axis illustrates the significant role of the gut microbiome, and its alteration, for instance by probiotics, may play in the development of acne. Modification of local and systemic inflammatory profiles by GI flora presents a target for potential therapy.

3. Pathophysiology of probiotics and acne

It is quite evident that the gut-brain-skin axis plays a theoretically significant role in the formation of acne lesions. In the following sections, we will discuss the pathophysiology behind probiotics and their ensuing potential impact in the arena of acne treatment. As previously discussed, the early theories introduced by Stokes and Pillsbury conceptualized the functional interdependence of the gut-brain-skin axis. It was further proposed that alterations in the neural axis result in gastrointestinal dysfunction, thereby disrupting the local normal flora, and resulting in widespread inflammatory response [37]. As we will see, the concept of systemic inflammatory response as well as oxidative stress is at the core of the rationale behind probiotics and their role in acne treatment.

3.1. Inflammation

The initial research during the era of Stokes and Pillsbury began with the discovery of concomitant hypochlorhydria in a significant portion of acne patients [37]. Additionally, the expanded SIBO theory suggested that an increased pH in the stomach resulted in a migration of bacteria proximally, increased gut permeability, and significant resultant inflammation [37]. This inflammation is the key starting point for the inflammatory cascade ultimately resulting in acne lesions.

The inflammatory state associated with acne has received much attention from dermatologic research studies and literature in recent years. While it was previously thought that events such as follicular keratinization and bacterial colonization preceded inflammation [38], it is now known that inflammation is actually the herald event [39].

The concept of acne as a result of inflammation is based upon the understanding that the immune system is designed to defend the human body against actual threats. However, in the acne patient, we are recognizing a chronic, low level of inflammation in the absence of threat [40]. Ideally, probiotics would eliminate this chronic inflammatory state, and in turn, halt the development of acne lesions.

Subclinical microcomedones are established as the earliest lesions of acne, and even at this early stage, inflammatory cells have been observed to be already present in these primary lesions. A modern research study was performed comparing immunohistochemistry and immunofluorescence of early, inflamed papules less than 6 h old in acne patients to both uninvolved skin of acne patients and a nonacne control group. In papules less than 6 h old, a remarkable increase in K16 and K67 activity is observed [39]. Uninvolved skin in acne patients exhibited increased expression of CD4+ T cells, and an even more significant upregulation of CD4+ T cells was observed in papules less than 6 h old [39]. The presence of macrophages was found to be higher in both uninvolved skin of acne patients as well as papules less than 6 h old of acne patients compared to nonacne controls [39].

One of the strongest pieces of evidence supporting the theory of baseline inflammation in acne patients is the increased presence of interleukin-1-alpha (IL-1), a well-known pro-inflammatory cytokine. In the above study, an increased level of IL-1-alpha was observed in both early lesional skin and uninvolved skin of acne patients in comparison with the control group [39]. IL-1 has been proposed as the signal that triggers the entire inflammatory cascade in the setting of a wound. In response to endothelial injury, IL-1 is the first cytokine to be produced, attracting lymphocytes to the area as well as activating endothelial cells to produce a hyperproliferative state [41]. It is further proposed that increased expression of K6 and K16, TNF-alpha, and endothelial growth factors then occur as a result [41]. Considering the above information, it may be deduced that IL-1 is a powerful inflammatory cascade that trigger in the setting of acne as well.

Collectively, several conclusions can be drawn from this information. These findings support the theory of acne as an inflammatory disease. Significant evidence reinforces the theory that inflammation precedes the overproduction of sebum, hyperproliferative state, and other physical manifestations of acneiform lesions. Taking into account the subtype of T cell activation observed, it is prudent to believe the inflammation is specific and antigenic in nature rather than an innate response [39].

Therefore, the anti-inflammatory actions of probiotics may be beneficial in the treatment of acne. Although the exact mechanism remains unclear, literature exists that suggests that *Lactobacilli* have been shown to modulate Th1/Th2 activity [42]. A separate study examined the Th1/Th2 inflammatory response of rats, when faced with an antigen challenge, in the setting of pretreatment with a combination of *Lactobacilli* and *Bifidobacterium* strains. It was found that the combination probiotic treatment did in fact alter both the Th1 and Th2 response [43]. As previously discussed in this section, a dysregulation of the T-cell response has been demonstrated in the skin of acne patients and it may be deduced that normalizing this response may be a critical step toward decreasing the baseline inflammatory state in this population.

Further solidifying this concept, in a study aimed at examining the immunomodulatory effects of probiotics in subjects with food allergies, it was determined that probiotics do in fact increase production of anti-inflammatory cytokines such as IL-10, TNF-α, and INF-γ [44]. This discovery may be re-enforced by looking back to the research involving rats and pretreatment with combined *Lactobacilli* and *Bifidobacterium*. In this study, significant reductions were also observed in the production of inflammatory cytokines, most notably IL-1α and IL-1β [43]. It should be noted that TNF-α production was decreased as well [43]. As previously discussed, the IL-1 cytokines play a key role upstream in the inflammatory cascade and altering the production of this cytokine via probiotics may prove advantageous in treating the acne patient.

Given the aforementioned research, it is once again reasonable to conclude that the addition of probiotics in the acne-prone patient would positively affect the causatory state of inflammation. It is clear that further research is needed to solidify the definitive effects of probiotics on the low-level inflammatory state and subsequent inflammatory cascade.

3.2. Oxidative stress

An alternative theory proposed by Allan L. Lorincz suggested that oxidative breakdown of lipids and squalene was a cause of acne rather than a consequence. The theory then goes on to suggest that this oxidative process is a trigger for the inflammatory condition seen in acne patients [38]. Subsequent studies reinforced this theory. In 1975, A Tappel also supported the theory of inflammation stemming from the damaging effects of lipid peroxidation [45].

This is both important and relevant in the setting of acne as squalene, a key component in the formation of the comedone, is sensitive to oxidative stress. In an independent study, squalene, when exposed to UV radiation (a source leading to oxidative stress), became increasingly comedogenic [46].

It has thus been proposed that alongside inflammation, oxidative stress may play a significant role in the development of acne lesions. Reactive oxygen species (ROS) are produced by environmental factors as well as cellular metabolism byproducts. Higher levels of ROS encourage an environment that is more hospitable to bacteria such as *P. acnes* [38]. In a study examining the activity of antioxidants defense enzymes in leukocytes, acne patients were found to have low levels of both superoxide dismutase and glutathione peroxidase [47].

Faulty antioxidant response seen in acne patients provides yet another role for probiotics in the treatment of acne. Probiotics have been proven to assist in antioxidant activity. In a study performed on the probiotic, *Bacillus coagulans RK-02*, evidence came to light that the bacteria produced a potent extracellular polysaccharide with significant antioxidant activity as well as superoxide radical scavenging activity and hydroxyl radical scavenging activity, even when measured against classic antioxidants including vitamin C [48].

In a separate study, researchers combined various strains of *Lactobacillus* with a gene encoding for superoxide dismutase. The *Lactobacilli* were found not only to successfully express the gene, but were also found to provide measurable defense against hydrogen peroxide species [49].

Probiotics provide a mechanism to counter free radical damage and increase antioxidant activity, resulting in an environment that is less attractive for *P. acnes* colonization.

4. Probiotics used for the treatment of acne

4.1. Oral probiotics

The idea of treating acne with probiotics dates back to the 1930s. During that time, *Lactobacillus acidophilus (*a common probiotic found in foods such as yogurt) was a popular diet supplement for the treatment of acne among the public [5]. Although this trend was widely accepted, formal research had not been carried out proving its effectiveness. It was not until 1961 that the first official clinical trial regarding probiotics and its relationship to acne was published. The trial was performed by a physician from the Union Memorial Hospital in Baltimore, Maryland named Robert H. Siver. Dr. Siver followed 300 patients who were taking a commercially available oral probiotic tablet called "Latinex" (combination of *L. acidophilus* and *L. bulgaricus*). Subjects ingested this supplement for eight consecutive days followed by a two-week break and then repeated the process. Over time, he noticed that 80% of patients with acne experienced clearing of their skin, especially in those with inflammatory acne lesions. Despite this study lacking a placebo group to compare results and having an unconventional probiotic dosing regimen, the findings did suggest a promising linkage between the intestinal flora and acne [50].

After Dr. Siver's research was published, other researchers became interested in a correlation between oral probiotics and acne. Two studies, both published in a non-English language journal, continued to demonstrate a connection. In 1987, an Italian article was published by Marchetti et al., 20 of the 40 patients with acne were given 250 mg of freeze-dried *L. acidophilus* and *Bifidobacterium bifidum* in addition to standard acne treatment. Subjects in the study group exhibited better compliance with their antibiotic regimen in addition to seeing improved clinical results in their acne [51]. In 2001, a similar investigation was performed in Russia by Vokova et al. using 114 subjects with acne. He found that 61% of the subjects had impaired bacterial microflora, and after probiotic supplementation in addition to combined acne therapy, their duration of treatment was greatly reduced to that of subjects without dysbacteriosis [22].

More recent studies have continued to confirm these results. In 2010, Kim et al. randomized 36 subjects with acne to receive either lactoferrin (a milk protein with anti-inflammatory, bactericidal, and fungicidal properties) added to fermented milk (experimental group) or fermented milk alone (control group). After 12 weeks, the experimental group experienced significant decreases in total lesion count (23.1%), inflammatory lesion count (38.6%), acne grade (20.3%), and sebum content (31.1%) compared to the control group. Although this study had the additional element of lactoferrin, both groups responded to the fermented milk and saw a reduction in total skin surface lipids. Furthermore, the addition of lactoferrin decreased a specific group of lipids called triacylglycerols, directly related to the decreased sebum content, acne lesion counts, and acne grade [52].

An interesting open-label study was published in 2013 by Jung et al. concerning probiotics versus antibiotics in 45 women between the ages of 18 to 35 years old. The females were randomized into one of three groups: probiotics only (a mixture of *L. acidophilus*, *L. delbrueckii*, and *B. bifidum*), oral minocycline only, or both probiotics and minocycline. After the first 4 weeks,

all patients observed significant improvement in their total lesion count; however, after 8 and 12 weeks, the group using both probiotics and minocycline experienced a significant decrease in their total lesion count compared to the other two groups. In addition, two subjects in the minocycline-only group developed vaginal candidiasis, an adverse event not observed in the group taking both. This study demonstrated that not only can probiotics augment antibiotic therapy, but they may also alleviate particular side effects experienced with chronic antibiotic use by suppressing the growth of unwanted organisms [53].

A 2016 clinical trial identified 57 patients with erythematous papulopustular facial rashes that were diagnosed as either acne, seborrheic dermatitis, or rosacea. The participants were started on a vegetarian diet and appropriate standard therapy for their disorder, including antibiotics, retinoids, and/or steroids. 37 patients of these patients were randomized to receive a daily oral probiotic supplement with *E. coli* Nissle. The group receiving probiotics showed an 89% improvement in their facial dermatoses compared to 56% improvement achieved with diet and standard therapy in the control group. In addition, white blood cell count via blood draw and immunoassays of IL-8, INF-α, and IgA levels were measured throughout the trial. After treatment, lymphocytosis disappeared by 78% in the probiotic group compared to 42% in the control group. Also, levels of INF-α, IL-8, and Ig-A normalized only in the probiotic group compared to no change seen in the control group [54].

4.2. Topical probiotics

With the growing body of evidence for the role of systemic probiotics in the treatment of acne, the efficacy of topical probiotics is also generating interest and investigation. Similar to oral probiotics, the use of topical probiotics dates back to the early 1900s [55]; however, proper clinical trials were not conducted until much later. In 1999, Di Marzio et al. completed the first clinical trial evaluating topical probiotics and their effects on ceramide production in the skin. Ceramides are waxy lipid molecules that comprise 50% of the lipid matrix within the intercellular spaces of the stratum corneum. Along with cholesterol and long-chain fatty acids, they are essential to maintaining the water permeability of the skin barrier. Ceramides have been found to be low in patients with aged skin, xerosis, atopic dermatitis, psoriasis, and even acne; therefore, increasing their production may significantly impact these disorders [56]. Initially, Di Marzio et al. conducted an *in vitro* study during which he added the bacterium *Streptococcus thermophilus* to human keratinocyte cell cultures and found an increase in the production of ceramides. He believed this was due to *S. thermophilus'* possession of sphingomyelinase, an enzyme that hydrolyzes sphingomyelin into ceramides. Many bacteria have been reported to produce extracellular sphingomyelinase including the genera *Bacillus, Listeria, Staphylococcus, Mycobacterium, Chlamydia, Pseudomonas, Leptospira,* and some species of *Helicobacter.* Although this enzyme primarily functions as a virulence factor for the bacteria, its ability to increase ceramide production may provide a benefit in treating skin diseases [57].

In the next phase of the study, Di Marzio tested this theory *in vivo* on 17 healthy subjects with normal skin. The subjects were instructed to apply 0.5 g of a topical probiotic formulation consisting of *Streptococcus thermophilus* twice a day to the volar surface of one of their forearms. They applied the vehicle alone to the contralateral forearm for comparison. An

additional four subjects were treated with sphingomyelinase purified from *Bacillus cereus* to ensure that the results produced were specific to the sphingomyelinase and not another component within the bacterium. After seven consecutive days of application, the probiotic formulation containing *S. thermophilus* caused an increase in the production of ceramides in the stratum corneum, which was comparable to the results seen using the sphingomyelinase extracted from *B. cereus.* These results demonstrated that the sphingomyelinase produced by *S. thermophilus* may improve skin barrier function [58].

Ceramides not only have a role in water permeability, but they also play a part in the antimicrobial and anti-inflammatory properties of the skin. The exact antimicrobial mechanism of ceramides has not been confirmed; however, there are many theories: reduction of bacteria adherence to epithelial cells, inhibition of bacterial protein kinases, and/or damage to the cell wall of the bacteria [59, 60]. Aware of their antimicrobial properties, Pavicic et al. performed a study in 2007 to evaluate the role of ceramides in patients with acne. The study consisted of both an *in vitro* and *in vivo* phase. *In vitro*, he found that phytosphingosine (PS), one of the four types of sphingoid bases that make up ceramides, inhibited growth of *Propionibacterium acnes*, an important contributor to acne formation. From these findings, he performed a two-part *in vivo* pilot study testing a 0.2% PS formulation on subjects with acne. In the first part, 30 subjects with acne applied a topical medication containing PS with benzoyl peroxide (PS-BPO) to half of their face versus benzoyl peroxide (BPO) alone to the contralateral side of their face two times per day. After 2 months, comedones were reduced by 72% and inflammatory papules and pustules by 88% in the PS-BPO group versus 22 and 32%, respectively, in BPO only group. In another arm of the trial, 10 subjects applied PS alone to half of their face and a placebo cream alone to the other side of their face twice a day. After 2 months, the placebo increased comedones by 43% compared to only 6% in the PS group. More significant results were seen in inflammatory acne numbers with an 89% reduction observed in the PS group compared to no change in the placebo group [61].

Topical probiotics may also help with stress-induced acne. It is known that acne can be exacerbated due to stress, primarily due to a release of a chemical called substance P. Sebocytes stimulated by substance P show higher levels of proinflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) tumor necrosis factor-alpha (TNF-alpha), and peroxisome proliferators activated receptors-gamma (PRAR-gamma), compared to a control [36]. Studies by Gueniche et al. in 2010, using both *in vitro* and clinical trials, have shown that two bacteria, *Lactobacillus paracasei* and *Bifidobacterium longum,* may improve inflammatory skin conditions by inhibiting substance P [33, 62]. As an adjunctive to current acne therapy, these two bacteria in topical formulations may provide relief for individuals suffering from inflammatory and/or stress-induced acne not responding to conventional treatment methods.

4.3. Future directions

Studies of topical and oral probiotics have demonstrated the beneficial anti-inflammatory and antibacterial properties of probiotics in the treatment of acne. Newer research is focusing on yet another treatment mechanism—the production of antimicrobial peptides (AMPs). AMPs are molecules produced by the innate immune system of a wide range of organisms, including

humans, plants, and insects, that act as a first line of defense against natural antimicrobial agents [63]. These peptides are extremely small, are anti-inflammatory, and have been shown to exhibit properties against bacteria, fungi, viruses, and tumors. They have even shown the ability to overcome bacterial resistance because it is difficult to develop complete resistance to AMPs, making them potential candidates for future therapeutic medications [64, 65]. Besides being produced by many eukaryotic organisms, numerous bacteria have been found to produce AMPs. These bacterial AMPs, called bacteriocidins, have been isolated from about 50 various bacterial species, especially lactic acid-producing bacteria [66–68]. Some researchers refer to bacteriocidins as only those produced by Gram-positive bacteria, which are further classified into two subgroups: lantibiotics (class I) and nonlantibiotics (class II). Many of the lactic acid bacteria produce AMPs in the nonlantibiotics group. AMPs produced by Gram-negative bacteria are sometimes referred to as microcins and classified further into two groups: class I and class II [68]. For simplicity, the general term "bacteriocidins" will be used here.

Compared to AMPs produced by eukaryotic organisms, bacteriocidins have a narrower spectrum of activity, only capable of targeting a few species but have the advantage of being more potent. Bacteriocidins are active at pico- to nanomolar concentrations compared to micromolar concentrations required when produced by eukaryotes. Bacteriocidins are bactericidal, causing pore formation in cell membranes [69].

There have been multiple studies performed observing the effects of AMPs on many disorders, including acne vulgaris. In 2006, Bowe et al. discovered that a normal oral flora bacterium, *Streptococcus salivarius*, was capable of inhibiting the growth of *P. acnes* by producing a bacteriocidin called bacteriocin-like inhibitory substances (BLIS). While BLIS is responsible for inhibiting group A streptococcus (GAS), a pathogenic bacterium responsible for causing many upper respiratory infections, its activity against *P. acnes* had not previously been evaluated. In this *in vitro* study, oral swabs were taken from 106 subjects and cultured for the growth of *S. salivarius*. Out of 106, 33 specimens yielded growth of *S. salivarius* and were available for assays of *P. acnes* and GAS. Results found 11 (33.3%) inhibited the growth of *P. acnes* and 13 (39.4%) inhibited the growth of GAS. Although these results focused only on *in vitro* activity, this study demonstrated the potential use of BLIS or BLIS-producing bacteria in future as acne topical treatment formulations [70].

A similar study in 2009 by Kang et al. demonstrated the effects of the bacterium, *Enterococcus faecalis* SL-5 (a very common inhabitant of the human gastrointestinal tract) and its effect on *P. acnes*. He conducted *in vitro* and *in vivo* studies. In the *in vitro* aspect of the study, *E. faecalis* proved to be bacteriocidal to *P. acnes* due to a bacteriocidin named ESL5. In the clinical trial, 70 subjects with mild-to-moderate acne were enrolled in an 8-week double-blind, randomized, placebo-controlled phase III study. Subjects were randomized into the probiotic or placebo group. Those in the experimental group applied a lotion containing ESL5 to the areas of the face involved with acne twice per day, and the control group applied a placebo lotion twice daily. After 8 weeks of application, a decrease in the number of comedones was seen in the probiotic group compared to the placebo group; however, these results were not statistically significant. In the inflammatory lesion counts, a statistically significant reduction of greater than 50% was observed in the *E. faecalis* group compared to placebo [71].

While these findings suggest that probiotics and the AMPs produced may benefit patients with acne, larger randomized controlled clinical trials are needed. Further studies will elucidate the most efficacious strains, preparations, and treatment regimens for the treatment of acne and potential uses in other conditions.

5. Conclusion

In summary, oral and topical probiotics are emerging as an exciting treatment option or adjuvant treatment for acne. Although additional research needs to be performed, the clinical trials conducted so far have continued to provide evidence that probiotics can improve acne, along with multiple other inflammatory disorders, with very limited adverse effects. In the upcoming years, probiotic formulations have the potential to be a fundamental component of acne treatment and may augment the efficacy of current treatments today.

Conflict of interest

No conflict of interest to be reported by the authors.

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Chapter 6

Lactobacillus Species in Breast Milk

Martin Gregora

Additional information is available at the end of the chapter

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Abstract

Lactobacillus species, present in the microbiota of breast milk, is a probiotic that deserves significant attention. It has a beneficial effect on the composition of the intestinal microflora and the intestinal immune system. In infants who were having *Lactobacillus fermentum*, a lower incidence of gastrointestinal and respiratory infections was noticed, in contrast to the control group. The significant anti-inflammatory effect of *L. fermentum* can be utilized to prevent and treat mastitis in breastfeeding women. It has also been shown to have a better clinical effect than classic antibiotics. Moreover, the higher share of *L. fermentum* in intestinal microflora of children with normal weight compared to obese ones opens other potential possibilities of the use of this probiotic.

Keywords: *Lactobacillus*, microbiota, probiotics, mastitis, obesity

1. Introduction

Microbiota is a substantial collection of genetic and bioactive materials responsible for building and regulating our defense systems. Bacteria and their intestinal microbial proportions modulate the immune system, greatly affecting the health and illness of an individual. Gastrointestinal flora is in close and continuous contact with epithelial and immune cells. This constant stimulation is essential for the development and functioning of the immune system [1]. These types of bacteria that colonize the guts of a newborn determine how the system develops, acting as an important antigenic stimulus for developing the immune response.

In the last 20 years, probiotics, bifidobacteria, *Lactobacilli*, microorganisms, and gastrointestinal flora, all of which can modulate the aspects of both natural and acquired immune responses in the host and thus affect human health, have become of prime importance. This importance is, of course, widely emphasized commercially. However, the actual effects and actions of individual probiotic strains vary, and it is very important to know what specific

probiotics are considered in order to determine their effects. Bacteria colonize vast areas of the mucous membranes and they are also present in important body fluids like breast milk. The mother's vaginal flora and breast milk are clearly among the most important sources of bacteria for the newborn. Varying studies have reported differing quantities of live bacteria in breast milk, but most studies report median numbers of 10^2–10^3 and a range of 10^1–10^7 colony forming units per ml of breast milk [2]. The infant who receives 300–700 ml of milk per day receives a large amount of these bacteria at the same time. The microbiota of milk, like that of mucous membranes, is individual and changeable. The probiotic bacteria present in mucous membranes and breast milk includes *Lactobacillus fermentum*. The expected pathway by which *Lactobacilli* is received into the milk is enteromammary transport through the dendritic cells [3]. This type of transport is still a controversial subject; however, various studies suggest that dendritic cells can pick up bacteria located in the intestinal lumen and transfer them to the lamina propria. Once the bacteria get inside the dendritic cells, they can penetrate the mammary glands and other mucosal surfaces.

2. *Lactobacillus* species and infectious diseases of infants

Respiratory and gastrointestinal tract infections are a significant problem for young children attending daycare centers or preschool, especially in the winter season. Common infectious diseases are facilitated by a general immaturity of the immune system and of the respiratory and gastrointestinal tract function [4]. An increased number of acute diseases translate into a significant financial burden for both the family and society. The increased costs are related to medical care visits and medication as well as to time away from work and/or for payment for someone to look after a sick child [5].

The most widely used probiotic species, which belong to the genera *Lactobacillus* and *Bifidobacterium*, have shown clinically significant benefits in the treatment and prevention of childhood diarrheal and allergic diseases in at-risk populations such as allergic families, hospitalized patients, or children in daycare centers. In a study in which *Lactobacillus reuteri* was administered for 3 months in 336 otherwise healthy children attending daycare centers, it was shown that during the administration and for the next 3 months, the number of episodes of diarrhea has significantly decreased [6]. The effects of probiotics in preventing respiratory tract infections are also receiving increasing attention. In accordance with the same study mentioned earlier, the number of respiratory tract infections in the 336 children has also significantly decreased at 3 and 6 months after the administration of the probiotics [6]. There are many sources of confusion concerning probiotic intervention in children. First, the mode of probiotic administration in the general child population is challenging. Second, the selection of a specific probiotic strain or a probiotic mixture is crucial for the possible beneficial effects. The duration of breastfeeding and the use of infant formula also affect the outcome [7]. Several clinical studies have been carried out to investigate bacteria isolated from human milk. In a 6-month study [8] with 91 infants in the control group and 97 infants in the *L. fermentum* group, a reduction in the total number of infections, especially gastrointestinal tract and respiratory infections, was observed in the probiotics group (**Table 1**). *L. fermentum* was selected for the study for safety and for its anti-infective and immunomodulatory properties.

	Control group	Experimental group	Incidence rate decrease (%)
Total infections	189	142	30
Gastrointestinal infections	33	19	46
Respiratory infections	134	106	26
• Upper respiratory	121	94	27
• Lower respiratory	13	12	13

Table 1. *Lactobacillus fermentum* administered to 6-month-old infants over a 6-month period versus *Lactobacilli*-free control group [8].

This strain is also able to colonize the mammary glands when administered to nursing mothers in capsule form. A similar effect on the health of children has been described in other probiotic strains. A multicenter, randomized, double-blind, placebo-controlled trial [4] on 126 healthy children aged 12–48 months with *Lactobacillus paracasei* (66 infants in the experimental group and 60 infants in the placebo group) showed a lower incidence of respiratory and gastrointestinal tract infections in the experimental group than in the control group (**Table 2**). An immunostimulatory effect was observed, consisting of a significant increase in the production of innate and acquired immunity peptides. Innate immunity peptides, produced by epithelial cells, Paneth cells, neutrophils, and macrophages, act as endogenous antimicrobial substances and defend the body against a broad range of pathogens (bacteria, fungi, protozoa, and viruses).

Another bacterium isolated from breast milk that has a positive effect on diseases in infants and children is *L. reuteri*. The mechanism of action of *L. reuteri* strains has been evaluated in *in vitro* and animal studies. One of the best-documented mechanisms is their antimicrobial activity. *L. reuteri* strains produce reuterin, a broad-spectrum antibacterial substance that can

Disease	Control group	Experimental group	*p*
Acute gastroenteritis, *n* (%)	24 (40.0)	12 (18.2)	0.007
(number of episodes)	(28)	(19)	
Rhinitis, *n* (%)	24 (40.0)	22 (33.3)	0.438
(number of episodes)	(50)	(44)	
Otitis media, *n* (%)	13 (21.7)	8 (12.1)	0.151
(number of episodes)	(17)	(11)	
Pharyngitis, *n* (%)	25 (41.7)	13 (19.7)	0.007
(number of episodes)	(30)	(22)	
Laryngitis, *n* (%)	14 (23.3)	6 (9.1)	0.029
(number of episodes)	(14)	(7)	
Tracheitis, *n* (%)	19 (31.7)	11 (16.7)	0.048
(number of episodes)	(30)	(16)	

Table 2. Common infectious diseases observed during the study period [4].

inhibit the growth of a wide spectrum of microorganisms such as Gram-positive or -negative bacteria, yeast, fungi, and parasites. *L. reuteri* strains may also regulate immune response. The results of 14 studies involving controlled trials and one systematic review indicate that the use of *L. reuteri* may be considered in the management of acute gastroenteritis as an adjunct to rehydration. There is also some evidence that *L. reuteri* is effective in reducing the incidence of diarrhea in children attending daycare centers [9]. *Lactobacillus rhamnosus*, a probiotic strain of human origin, also influences immune response both specifically by stimulating antibody production and non-specifically by enhancing the phagocytic activity of the blood leucocytes. It can promote the recovery from rotavirus diarrhea and can reduce the incidence of diarrhea associated with the use of antibiotics. In a randomized, double-blind, placebo-controlled study with 571 healthy children aged 1–6 years, there was a 17% relative reduction in the number of children with respiratory infections with complications and lower respiratory tract infections and a 19% relative reduction in antibiotic treatments for respiratory infection [10].

3. *L. fermentum* in the treatment of mastitis

Mastitis is a common disease during lactation, affecting 3–33% of lactating mothers. Inflammation of the mammary glands usually has an infectious origin involving staphylococci, streptococci, and/or Corynebacterium. Traditionally, *Staphylococcus aureus* has been considered the main etiological agent of acute mastitis, although *Staphylococcus epidermidis* is emerging as the leading cause of chronic mastitis. Multidrug resistance and/or the formation of biofilms are very common among clinical isolates of these two staphylococcal species. This explains why mastitis is difficult to treat with antibiotics and why it constitutes one of the main reasons to cease breastfeeding. In this context, the development of new strategies based on probiotics, as alternatives or complements to antibiotic therapy for the management of mastitis, is particularly appealing. The anti-inflammatory effect of *L. fermentum* can be successfully used to prevent and treat mastitis in a breastfeeding woman. Given as a nutritional supplement to a woman with breast inflammation, it demonstrated a better clinical effect than conventional antibiotics. Moreover, a higher proportion of *L. fermentum* in breast milk is beneficial to the child by favorably modulating the child's intestinal microflora, with beneficial consequences for the immune system and health. A study [11] of 352 women with symptoms of mastitis demonstrated a beneficial effect of treatment with lactobacilli. The women were divided into three different groups: one group using *L. fermentum* and one group using *Lactobacillus salivarius*, both strains isolated from human milk and a third group receiving antibiotics. After 21 days, a reduction in the number of the main etiological agents causing mastitis (*S. epidermidis*, *S. aureus*, and *Streptococcus mitis*) was observed. This reduction was greater in the probiotic groups (**Figure 1**). The groups in which *Lactobacilli* were used also experienced greater pain reduction (**Figure 2**). A similar study of 225 women with severe mastitis caused by staphylococci demonstrated a beneficial effect of *L. fermentum* treatment. There was a faster retreat than in the control group treated with antibiotics. Mastitis relapse was more common among the women treated with antibiotics (31% versus 10%). The principle of the antibacterial action of *L. fermentum* could be explained by its high ability to adhere

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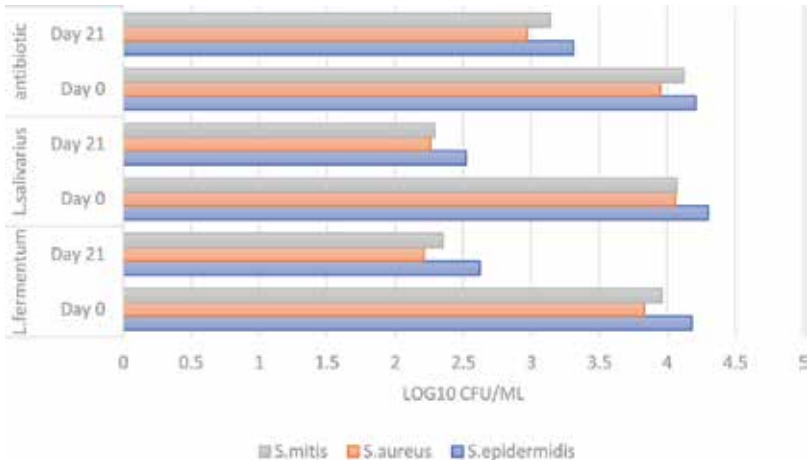

Figure 1. Bacterial counts from breast milk at the beginning (day 0) and at the end (day 21) of the trial [11].

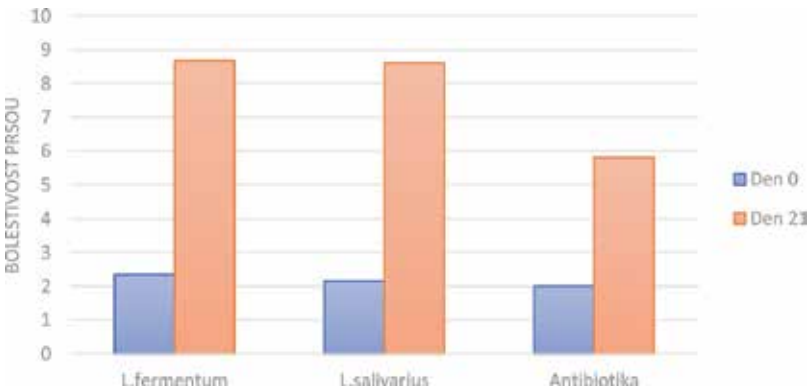

Figure 2. Breast pain score at the beginning (day 0) and at the end (day 21) of the trial. Pain is expressed as extremely painful (0) to no pain (10) [11].

to epithelial cells and inhibit the adhesion of pathogenic bacteria by producing antimicrobial compounds (lactic acid, H_2O_2) and by its effect on increased mucin production. The action of *L. fermentum* is immunostimulatory.

4. Individually different microflora of normal-weight and obese individuals and the role of probiotics

Obesity is viewed as one of the more important public health problems of our time, and the velocity of propagation is highest in children. This can lead to a vicious circle: obese children often become obese adults, and maternal obesity overnourishes the fetus, thereby programming adult size and health with a heightened risk of obesity later in life. Recent scientific

advances point to systemic low-grade inflammation and local gut microbiota as contributing factors for overnutrition. The gut microbiota enables the hydrolysis of indigestible polysaccharides into easily absorbable monosaccharides and the activation of lipoprotein lipase by direct action on the villous epithelium. Consequently, glucose is rapidly absorbed and fatty acids are excessively stored, with both processes boosting weight gain.

Bacterial milk composition in obese mothers differs from the bacterial milk composition of mothers with standard body weight [11]. Since breast milk is one of the most important means of colonizing infants with bacteria, there is an idea that there is a relationship between obesity and the transmission of microbial flora from mother to infant. It is known that obese infants and obese children generally have very different microbial flora from infants who are lean and healthy (**Figure 3**). The results reported by Kalliomäki et al. suggested that gut microbiota deviations predispose individuals toward energy storage and obesity. The genus *Bifidobacterium*, affecting both the quantity and quality of the microbiota during the first year of life, was shown to be higher in children who remained normal weight than in children developing overweight. The microbiota aberrancy during infancy in children becoming overweight was also associated with a greater number of *S. aureus* than in children remaining normal weight as assessed by real-time qRT-PCR. These findings imply that high numbers of probiotics and low numbers of *S. aureus* in infancy may provide protection against overweight and obesity development.

Perhaps it would be advisable to think about intervention in cases of obese mothers. When is the right time for such an intervention? We know that some bacteria are transmitted from mother to infant. For an obese mother, it would be most helpful to choose an appropriate intervention before or during pregnancy, in any case before giving birth. If the microbial flora has already been transferred to the infant, it could be optimized during breastfeeding through specific probiotics. *L. fermentum*, a strain isolated directly from breast milk in the form of a food supplement, is available as a possible solution. Whether the expected effect of normalization of the intestinal microflora can be produced by such a solution should be confirmed by further studies.

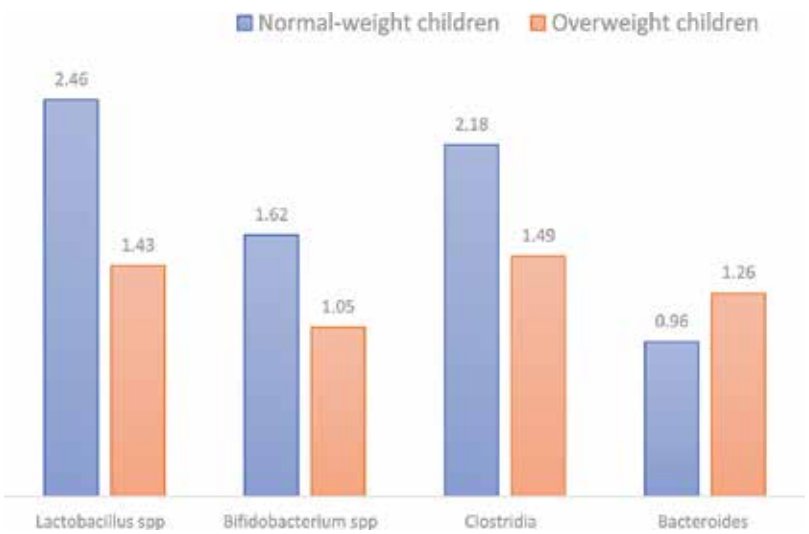

Figure 3. Bacterial counts in fecal samples analyzed by fluorescent *in situ* hybridization during infancy (6–12 months) [12].

5. The effect of probiotics deserves further clinical trials

The mucosal microbiota is formed by millions of bacteria. The *Lactobacillus* species are undoubtedly important bacteria for the development of humoral and cellular immunity. However, in the human gut, they are only a part of a huge mosaic where each particle has its place and function. After decades of research, probiotics are still an open chapter of great and unimagined opportunities to influence the immune system and to treat some of civilization's diseases. Most of these diseases are multifactorial. Influencing the mucosal microflora seems to be a promising step. Available data suggest that some probiotics such as *L. fermentum*, *L. reuteri*, *L. paracasei*, and *L. rhamnosus* may have some effect on community-acquired infections; however, confirmation studies are still needed.

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Chapter 7

Probiotics Consumption Increment through the Use of Whey-Based Fermented Beverages

Mónica S. Molero and Wilfido J. Briñez

Additional information is available at the end of the chapter

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Abstract

Probiotics have been taking value over the last years due to its benefits in human health. Researchers have been looking for options in order to increase probiotics consumption, and one of the more nutritional choices is to use whey as a substrate in fermented beverages. Whey is a by-product liquid obtained during cheese processing. It is an economic source of protein, which provides multiple properties in foods. The main objective of this chapter was to carry out a complete review of important researches related to whey-based fermented beverages production. Researches show that probiotic micro-organisms have the ability to grow in whey properly, in such a way that they reach high concentrations, needed to achieve the probiotic effect that consumers are looking for. Certain substances, such as fruit pulps and carboxymethyl cellulose, have been used to improve viscosity, flavor among other important characteristics. Sensorial evaluations have been performed in order to assess consumers' impression, and they have been pleasantly accepted. Average shelf-life is 21 days. Through this review, it is known that whey is an excellent alternative to increment probiotic consumption, not only because it is an outstanding substrate for probiotic micro-organism's growth but also due to its excellent sensorial characteristics.

Keywords: whey, fermented beverage, acid lactic bacteria, probiotic, organoleptic characteristics

1. Introduction

During the last decades, the use of probiotics has been increasing, due to their important benefits in human health. Kollath, in [1], first defined the term "probiotic," when he suggested the term to denote all organic and inorganic food complexes as "probiotics," in contrast to harmful antibiotics, for the purpose of upgrading such food complexes as supplements. In

1965, Lilly et al. [2] used the term "probiotic" to describe those substances secreted by an organism that stimulate the growth of another. Since then, this definition has been evolving remarkably, so that today, probiotics are defined as microbial dietary supplements, viable, selected, which when are introduced in sufficient amount, affect human organism beneficially through their effects on the intestinal tract [3]. On the other hand, Vasudha and Mishra [4] define them as alive microbial supplements, which beneficially affect the host by improving its intestinal microbial balance.

Dairy products have become a healthy alternative to increase probiotics consumption, developing fermented beverages based on milk, whey or their mixture. Whey has been less used than milk. However, it has wonderful physico-chemical characteristics that make it an excellent substrate to be used in the development of fermented beverages.

Whey is a green translucent liquid obtained by separating milk clot in cheese making process [5]. Its composition and characteristics depend on the technological process used and the type of milk. It is composed of 5% lactose, 93% water, 0.85% protein, 0.53% minerals, and 0.36% fat [6].

Its characteristics correspond to a fluid of yellowish green color, turbid, fresh taste, weakly sweet, acidic, with a content of nutrients of 5.5–7% that come from milk. It retains about 55% of total milk ingredients like lactose, soluble proteins, lipids, and mineral salts [7]. Whey is a by-product of high energetic and nutritional quality. For human being, it serves as an important source of vitamins, proteins, and carbohydrates.

Some statistical studies indicate that a significant portion of this waste is discarded to tributaries, resulting in an environmental problem due to its high biochemical oxygen demand. It physically and chemically affects the soil structure, decreasing the yield of agricultural crops, and polluting water because it depletes dissolved oxygen [7].

For the reasons explained above, dairy industry has been looking for alternatives for the use of this by-product, which is a high pollutant; however, it has a great nutritional value. Among the products of successful acceptance are fermented dairy drinks, refreshing beverages [8], protein concentrates [9], infant formulas [10], and others.

The processing of whey for beverages production began in the 1960s, and Rivella was the first fermented drink prepared from whey, made in Switzerland [11]. Whey products improve texture, reduce flavor and color, emulsify, stabilize, improve flow properties, and show many other functional properties that increase the quality of the products [12].

The main objective of this chapter is to carry out a complete review about fermented beverages based on whey inoculated with probiotics micro-organisms that have been produced around the world over the last years, focusing specially in important aspects such as sensorial and microbiological quality, shelf-life, and probiotic effects, showing that probiotics consumption can be increased through the use of whey as a substrate in this type of formulation, promoting it as a useful dairy by-product due to its excellent sensorial characteristics and its contribution in high quality organoleptic foods.

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2. Relevant aspects related to the use of whey as a substrate for the fermented beverages formulation

2.1. Whey physico-chemical characterization

The knowledge of whey physicochemical characterization is an important step in the use of this by-product in the dairy industry for different industrial processes. For this reason, most of the studies related to the use of whey propose a physico-chemical characterization in order to evaluate whether it meets the standards required to be used in technological processes.

In a very recent research, Molero et al. [13], carried out a physico-chemical characterization of whey obtained by cheese making process applying an artisanal method. It consisted of the determination of pH, titrated acidity, total solids, fat, protein according to the Venezuelan Standard COVENIN, and determination of lactose and minerals by analytical difference. The values obtained were statistically analyzed using a statistical package. The results classify whey as sweet, with excellent nutritional characteristics and attractive to be used in food technology for probiotic production, protein-fermented beverages, among other applications.

In an interesting research, Tirado et al. [14] carried out a physico-chemical characterization of whey derived from the production of coastal cheese. Fat analyzes were performed by Gerber method, lactose by the Lane and Eynon method (AOAC 923.09, 920.183b); the protein was analyzed by the Kjedahl method (AOAC 920,152); total solids by spectrophotometry; pH was determined by the method established in AOAC 945.10/90 and the acidity expressed as a percentage of lactic acid according to the Colombian Technical Standard. The values obtained were: fat 0%, lactose 3.69%, protein 2.29%, total solids 6.28%, acidity 0.08% lactic acid, and pH 6.5.

Linares et al. [15] showed similar results in the physico-chemical characterization of sweet whey samples, obtaining a pH of 6.84; acidity titrated of 0.11% (% lactic acid); protein between 0.6 and 1%; and ash 0.6%. De Paula et al. [16] performed a physico-chemical characterization of whey obtained from the manufacture of coastal cheese. This characterization was carried out using the following methods: acidity (AOAC 947.05/90), pH (AOAC 981.12/90), soluble solids (AOAC 932.12/90), total solids (AOAC 925,105/90), and lactose (FIL 28a/74). The following results were obtained: acidity (% lactic acid) 0.11; pH 6.58; total solids 6.83%, protein 0.98%; fat 0.4%; and lactose 4.54%.

Similarly, Montero et al. [17] carried out a whey fermentation with *Lactobacillus (L)* for feeding calves in the tropics. Researchers performed a physico-chemical characterization, determining pH, total protein, fat and total solids, tested by the Standard Method for examination of Dairy Products, 2004. The values obtained for these characteristics were in accordance with the Colombian standards established for this type of analysis.

In other studies, Londoño et al. [18] developed a fermented drink of fresh cheese whey inoculated with *L. casei*. Acidity, pH, viscosity, total solids, protein, fat, ash, lactose, minerals, soluble solids, reducing sugars, and moisture were determined for this purpose. Acidity, pH,

total solids, protein, fat, ash, soluble solids, were determined using AOAC methods; lactose was determined using the Teles reagent method; reducing sugars by the method of 3-amino-5-nirosalicylic acid; mineral content was determined by spectrophotometric method of atomic absorption and viscosity by the Brookfield method. The values obtained were found within the established standards for these parameters.

On the other hand, Miranda et al. [19] carried out a physico-chemical characterization of sweet and acid whey produced in the cheese complex of Bayamo (Cuba). The authors determined acidity, pH, density, and fat content, following the guidelines of the Ministry of Agriculture of Cuba; lactose was determined by the phenol-sulfuric method; dry extract, crude protein, calcium and phosphorus were tested according to internationally recommended methods. The acid whey was distinguished by a lower pH and a higher acidity than the sweet. No significant differences were observed between the two varieties of whey for the remaining characteristics tested. All of them were within the specifications of quality established by the Cuban norm. Low acidity of whey benefits its quality, because it allows a better use for human and animal feeding. It is great important to know about dry extract in the evaluation of the quality of cheese whey as raw material, because it would indicate its water content: a greater amount of water makes whey has less nutritional value.

In this order of ideas, Sepúlveda et al. [20] developed a fermented beverage with the use of fresh whey with the addition of Maracuyá pulp. For this purpose, a physico-chemical characterization was performed, determining pH, viscosity, total solids, protein content, fat, and ash by AOAC methods; lactose was determined by the reactive method of Teles; calcium, sodium, and potassium were determined using the spectrophotometric method of atomic absorption. When comparing composite ranges obtained with data reported by Amiot et al. [21], Scott [22], Posati and Orr [23], and Morales et al. [24], similarities were observed with measures reported for total solids, lactose and protein; under these levels of composition a substrate for fermentation was guaranteed, influencing in the performance of beverage processing properly. On the other hand, fatty content in whey is directly linked to the cheese manufacturing conditions. The values obtained in this trial were considerably lower than that reported by Scott [22], who states that fat content for sweet whey ranges from 0.2 to 0.7%. The pH obtained was slightly higher than that reported by Spreer [25] for this type of whey.

From the studies explained above, it is a fact that whey's physico-chemical characteristics vary depending on the composition of the milk, the cheese making process and the type of cheese, which could determine the ultimate destination of this by-product.

2.2. Microbial cultures used to produce fermented beverages based on whey

The cultures most likely used are lactic acid bacteria (LAB), which play an important role in fermentation processes. They are widely used in food industry because of their involvement in texture, taste, smell and aroma, and development of fermented foods [26].

LABs may be contained in a group of micro-organisms named lactic cultures or starters [27]. They are used in dairy industry for fermented milks production, cheeses, butter, and other products that are required to be fermented [28]. LABs were referred to as probiotics in the 1960s.

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The scientific interest in bacteria as protective agents against different diseases comes from the observation of Metchnikoff, who at the beginning of the twentieth century, emphasized the longevity and good health of the Bulgarian peasants, who consumed large quantities of yoghurt [29].

The observations of multiple scientists such as Trapp et al. [30] assumed that consumption of large quantities of foods rich in lactic acid bacteria, eliminated toxin-forming bacteria, while raising the proportion of lactic acid bacteria and intestinal flora, improved health and increased life expectancy. Since then, and throughout almost a hundred years of study, various authors have endeavored to know different functions of beneficial micro-organisms that populate the digestive tract.

Lilly et al. [2] used the term "probiotics" to describe those substances secreted by an organism that stimulates the growth of another, as opposed to the term "antibiotic," understood as any chemical compound used to eliminate or inhibit the growth of infectious organisms. Parker [31] was the first to use "probiotic" referring to organisms and substances that contribute to intestinal balance.

The definition of probiotics has evolved remarkably, so that today, they are defined as viable, microbial selected dietary supplements that, when they are introduced in sufficient amounts, affect the human organism through their effects on the intestinal tract [3]. Probiotics must meet some basic requirements to be selected in the development of commercial probiotic products. The most important requirements are: the probiotic micro-organism survives in the product, the physical and genetic stability during product storage is guaranteed, and all its essential properties that evidence its health benefits after consumption, are maintained during manufacture and storage of the product [32]. Laws et al. [33] states that the essential criteria for primer selection include acidification, aroma, taste, stability, and texture.

Many researches have been carried out with this class of micro-organisms, producing drinks of high microbiological and sensorial quality. Following the same idea, Molero et al. [34] formulated a probiotic fermented beverage based on whey, using a mixed culture of *L. acidophilus* and commercial yoghurt culture: *Streptococcus (S) thermophilus and L. bulgaricus*. Tirado et al. [14] produced a fermented whey milk drink using *S. salivarius* ssp. *thermophilus* and *L. casei*.

On the other hand, Linares et al. [15] evaluated the effect of different proportions of citrus pulp on the sensorial acceptability of a fermented and protein drink made from residual whey. For this purpose they used a lyophilized lactic culture of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. casei*. Likewise, Martínez et al. [35] formulated a fermented cheese-whey drink adding maracuyá pulp. For this purpose, lactic ferments were used for direct inoculation: *S. thermophilus*, *L. delbrueckii* sub *bulgaricus*, and *Lactococcus lactis* sub *lactis.* Similarly, Vela et al. [36] developed a probiotic whey-based fermented beverage with mango pulp and almonds, using isolated colonies of *L. casei.*

In another study, Fiorentini et al. [37] evaluated the influence of different combinations of probiotic bacteria and different fermentation temperatures on the physico-chemical characteristics of fermented lactic beverages based on soybean and whey. For this purpose, a lyophilized probiotic culture was used, composed of *L. acidophilus* and *Bifidobacterium (B) bifidum*. A second culture was prepared with *B. lactis* and *S. thermophilus*.

In other studies, Pescuma et al. [38] developed fermented functional beverages based on whey, using lactic acid bacteria: strains of *L. acidophilus*, *L.* subsp. *bulgaricus*, and *S. thermophilus*. A similar approach was given by Legarová and Kousimska [39] who formulated a whey-based drink using the same starter culture.

Katechaki et al. [40] performed a research related to thermal drying of *L. delbrueckii* subsp. *bulgaricus* and its efficient use as starter culture in whey fermentation and in cheese process. This micro-organism is a thermophilic LAB, able to ferment lactose, glucose, and fructose [12]. It was isolated from Bulgarian yoghurt, from Germany. Montero et al. [17] used *L. casei* to ferment whey to feed calves, the culture contained 1×10^7 CFU/ml. De Castro et al. [41] evaluated the effect of the incorporation of oligofructose on the properties of fermented probiotic lactic beverages using a probiotic culture composed of *L. acidophilus*, *B.*, and *S. thermophilus*. Oligofructose is an oligosaccharide, obtained from the enzymatic hydrolysis of insulin [42]. It is a prebiotic whose use can bring functional benefits and can affect the sensory properties of the products significantly [43].

Similarly, Londoño et al. [18] worked on a fresh cheese fermented drink formulation, inoculated with *L. casei* as a probiotic culture. Other cultures used were *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*.

In another research, Gallardo et al. [44] evaluated taste and sensation in the mouth of beverages made from whey with addition of hydrocolloids. The functionality of hydrocolloids at low concentrations is that it enhances viscosity and prevents particles sedimentation. It also contributes to the microstructural properties of meals, based on its ability to confer structure to the continuous phase of the substrate, which depends on their solubility in water and/or their intermolecular associations [45]. Beverages were prepared with a commercial yoghurt starter culture, which consisted of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*.

Hernández et al. [46] worked on the preparation of a probiotic drink based on whey, using cultures of *L. reuteri* and *B. bifidum*. The first was preserved on LBS (*Lactobacillus*) agar at 4°C. Three subcultures were performed consecutively prior to the use of the strain in the experiment with 1% inoculum. The second was preserved in MRS medium. Two subcultures were performed before use the strain in the experiment with 1% of inoculum.

Following the same idea, Dalev et al. [47] evaluated the sensory quality of whey-based probiotic beverages. A probiotic culture was prepared with strains of *B. breve* ATCC 15700, *B. infantis* ATCC 15697, *B. animalis/lactis* J38, *L. plantarum* W42, *L. plantarum* IB, *L. casei Lc* and *S. thermophilus*.

Sepulveda et al. [20] prepared a fermented beverage with the use of fresh whey with the addition of Maracuyá pulp, using a traditional lactic acid culture, in a 1:1 ratio of *S. thermophilus* and *L. bulgaricus*. Oliveira et al. [48] developed a fermented lactic drink using four probiotic cultures using *S. thermophilus and L. delbrueckii* ssp. *bulgaricus*; *L. acidophilus* and *L. rhamnosus*.

Kéfir has also been used as a starter culture in the production of beverages from whey [49–51]. Kefir is made by inoculating milk with kefir grains. This grain is irregular and its size varies

from 3 to 35 mm in diameter, contains lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*), acetic acid bacteria, and yeast mixture, coupled with casein and sugar through a matrix of polysaccharides called kefirán [52].

Yeasts, such as *Kluyveromyces marxianus*, have also been used as crops. This yeast has been isolated from fruit, cheese, yoghurt [53], milk [54], and has been used in whey processing. It has the ability to hydrolyze lactose and ferment sugars efficiently [55]. Cóndor et al. [56] prepared a beverage from cheese whey using immobilized *Kluyveromyces Marxianus* cells. Padín and Díaz [57] worked on the alcoholic fermentation of whey, using this yeast and organic solvents as extractants. Dragone et al. [58] performed the characterization of volatile components in an alcoholic beverage produced from whey with *Kluyveromyces marxianus* ATCC22.

2.3. Technological process followed for whey-based probiotic fermented beverages production

Table 1 summarizes the review of whey-based fermented beverages. It tells the technological process used in drinks manufacture. It is interesting to observe how the use of probiotic micro-organism plays an important role. The tendency is to use this type of bacteria and the reason is its potential benefit to human health.

In the development of these beverages, authors have used additives in order to improve some organoleptic characteristics, for example oligofructose, hydrocolloids, processed fruits, and others. Regarding technological process, fermentation is the essence of the drink production. However, it can be changes related to raw material (e.g., whey powder, liquid whey, combination with soymilk or whole milk), prior bacteria isolation, among others. Dairy industry has thus diversified methods for producing whey-based beverages. In the following sections, the acceptance of these drinks can be evidenced based on the sensorial evaluations and their probiotic character based on the viable count.

2.4. Fermented whey-based beverage sensorial quality

In the process of making dairy drinks, it is essential that they have adequate sensory properties to ensure they are accepted by consumers. Sensory quality researches have been increased over the recent years.

In this order of ideas, Molero et al. [59] carried out a sensory evaluation of probiotic fermented beverages based on whey. Four treatments were developed using combinations of two stabilizers, carboxymethyl cellulose and unflavored gelatin and two starter cultures: *L. acidophilus* and a mixed culture with *L. acidophilus* and yoghurt micro-organisms. For these, a sensory evaluation in three phases was designed. In a first phase test, acceptance-rejection was applied to an untrained 30 people in order to select the essence of fruits that best suited to drinks. In second place, preference test was performed with three concentrations of sugar, applied to 30 people panel. In a third phase, smell, taste, overall acceptance, and consistency of the four treatments were evaluated using an acceptance degree test with 5-point hedonic scale to an untrained panel of 100 people. Fruit essence largely accepted by the panelists was the coconut.

Author	Micro-organism	Special additives	Fermentative process
Molero et al. [13, 34]	*Lactobacillus acidophilus* and commercial yoghurt culture		Whey was pasteurized at 65°C for 20 min and cooled to 38°C. Inoculation of micro-organisms. Fermentation was carried out controlling pH until 4.5
Tirado [14]	*Streptococcus salivarius* ssp. *thermophilus y Lactobacillus casei* ssp. *casei*		Mixture of whey, skim milk powder and sugar was pasteurized at 63°C for 30 min and cooling to 43°C. Inoculation of micro-organisms in equal proportions. Fermentation was carried out for 3 h at 40°C controlling pH up to 5.
Linares et al. [15]	Lactic culture: *Streptococcus thermophilus*, *Lactobacillus delbrueckii* sub. *bulgaricus*, and *Lactobacillus casei*	Citrus fruit pulps	Fruit juice mixed with whey, previously pasteurized at 70°C for 30 min and fermented at 42°C for 5 h. White sugar was added in order to standardize at 14 °Brix. The beverage obtained was pasteurized at 80°C for 15 s and packed at the same temperature
Martínez et al. [35]	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* sub. *bulgaricus y Lactococcus lactis*	Pasteurized maracuya pulp	Five different treatments with different Maracuya pulp percentages (5; 7,5; 10; 12 y 15%) and final value of 14 °Brix
Vela et al. [36]	*Lactobacillus casei*	Mango pulp (*Magnifera indica* var. *Tommy atkin*) and almonds (*Amygdalus communis*)	150 ml of pasteurized whey was taken and inoculated with *Lactobacillus casei*. They were incubated at 35°C for 48 h. pH was measured. Fully mature pulp was added in a 1:1 ratio. 12.5% (w/v) of previously crushed almonds were added
Teixeira et al. [51]	Kéfir grains		Kefir grains washed with distilled water and inoculated in 250 ml of whey, with a temperature of 25°C for 72 h
Fiorentini et al. [37]	Probiotic freeze-dried cultured of *L. acidophilus* y *Bifidobacterium bifidum*	Water-soluble soybean extract	Whey powder was dissolved in water in a 1:1 ratio right before use. Mixture of 40% whole milk, 30% whey of mozzarella cheese, 30% water-soluble extract of soybean and 10% of sugar. Heat treatment at 90°C for 5 min, cooling to fermentation temperature (37°C). Incubation until reaching a pH between 4.5 and 5. Cooling at 20°C, homogenized, distributed in plastic bottles and stored at 7°C for 21 days
Legarová et al. [39]	Commercial yoghurt starter culture: *L. delbrueckii bulgaricus* y *Streptococcus thermophilus*		Incubation at 43°C and cooling at 4°C.
Katechaki et al. [40]	*L. delbrueckii bulgaricus*	*Lactobacillus delbrueckii* subsp. *Bulgaricus drying*	Drying was carried out in convection ovens at 35, 45, and 55°C for 10 h. After drying remaining moisture was removed with further drying at 102°C. Fermentation was performed at 37°C for 3 days

Author	Micro-organism	Special additives	Fermentative process
Pescuma et al. [38]	*L. acidophilus*, *L. bulgaricus y Streptococcus thermophilus*		Incubation a 37°C for 24 h
Padín and Díaz [57]	*Kluyveromyces marxianus*	Organic solvents	Reconstituted whey powder at 20% w/w. 100 ml of reconstituted whey 10% v/v were inoculated with a *Kluyveromyces marxianus* culture, incubated at 30°C. After 8 h 100 ml of selected solvents were added (oleic acid and soybean oil) in a ratio of 1:1 by maintaining them under the same experimental conditions for 30 h
Montero et al. [17]	*L. casei*		100 ml of culture was taken to inoculate 900 ml of whey in a beaker and incubated for 24 h at 39°C. Fermented whey was poured into 9 l of fresh whey and fermented 24 h at 39°C in a convection oven. The 10 l of fermented whey were poured into 90 l of fresh whey and rested 24 h at room temperature
De Castro et al. [41]	*L. acidophilus, Bifidobacterium* y *Streptococcus salivarius thermophilus*	Oligofructose	Pasteurized milk with commercial sucrose was heat treated at 95°C for 5 min while liquid whey with oligofructose was heated to 65°C for 30 min. The temperature of the mixture was lowered to 40°C. The beverage was made with the addition of the lyophilized culture at 8.3 mg/100 ml. Fermentation occurred at 40°C. pH of 4.6 was monitored. The beverage was then cooled to 4°C
Londoño et al. [18]	*L. casei*. Other cultures: *L. delbrueckii bulgaricus* and *Streptococcus salivarius thermophilus*	Inverted sugar syrup; maracuyá pulp; carboxymethyl cellulose (CMC)	Inoculation was performed maintaining a pH of 5.8 and stirring for 3–5 min. Subsequently, the beverage was flavored with the addition of maracuya pulp, packed and stored at 4°C
Hernández et al. [46]	*L. reuteri* y *Bifidobacterium bifidum.*		Reconstituted whey (7%) with addition of 7% sucrose and 0.4% pectin. Three treatments were applied by inoculation of probiotic strains in different ratios. Incubation at 37°C and storage at 4°C for 30 days
Gallardo et al. [44]	Commercial yoghurt starter culture: *L. delbrueckii bulgaricus* and *Streptococcus thermophilus*	Hydrocolloids	Fermentation was carried out by inoculation (2% v/v) with the starter culture at 42°C until reaching a pH of 4.6
Dalev et al. [47]	*Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium animalis/lactis*, *L. plantarum*, *L. casei*, and *Streptococcus thermophilus*	Soy milk	Equal amounts of whey and soy milk. Fermentation at 37°C for 24 h until reaching a pH of 4.4–4.6. Drinks were cooled and supplemented with processed fruits

Author	Micro-organism	Special additives	Fermentative process
Oliveira et al. [48]	Probiotics cultures: yoghurt culture of *Streptococcus thermophilus* y *L. delbrueckii bulgaricus*; *L. rhamnosus*		Complete and skimmed pasteurized milk mixed with skim milk powder to obtain 130 g/l of total solids and 26 g/l of fat, supplemented with 20 g/l of casein hydrolyzate. Heat treatment at 90°C for 10 min cooled to 4°C and stored 24 h prior to use. Fermentation with incubation at 42°C until reaching a pH of 4.3
Sepulveda et al. [20]	*Streptococcus thermophilus* y *L. bulgaricus.*	Maracuyá pulp (*Passiflora edulis*) and Carboxymethyl cellulose	It was incubated 2 or 3 h, maintaining the temperature until reaching a pH of 4.6. Fermentation was stopped with a fast cooling of 4°C. Agitation to ensure that maracuya pulp and vitamin dosage were well incorporated into the mixture
Cóndor et al. [56]	*Kluyveromyces marxianus*		Fermentation at 30°C for 7 days, evaluating the fermentation kinetics with the reading of °Brix and pH

Table 1 Whey-based fermented beverage technological process review

The preferred concentration of sugar by the panelists was 6%. The best evaluated treatments for consistency and general acceptance were those who had carboxymethyl cellulose as a stabilizer. The best drink evaluated in reference to the taste was the one containing carboxymethyl cellulose and *L. acidophilus*. The four drinks were equally qualified in relation to the smell.

Similarly, Valencia et al. [60] carried out a sensorial evaluation to nutritional drinks based on pumpkin and whey, enriched with oats and passion fruit. They evaluated 12 beverage formulations considering color, aroma, taste, and acceptability. They performed the test with a panel of 26 school-aged children; each child received three 100 mL samples. They used a questionnaire with expression faces of pleasure or displeasure, corresponding with a hedonic scale of 1–5, being 1 the lowest score and 5, the highest score. They found significant differences between the results obtained for the samples analyzed, but all of them were very well accepted by the panelists.

De Paula et al. [16] performed a sensory evaluation of fermented beverage fermented from whey with and without Maracuyá pulp. They performed an order-preference test, using a panel of 59 consumer catheters, using note 1 for the most preferred and 5 for the least preferred. They coded the samples and presented them randomly in 50 mL beakers, finding that the combination of whey with passion fruit flavor was the most preferred beverage. In addition, they indicated that the panelists described the product as very good, novel, and interesting.

In another research, Vela et al. [36] evaluated a probiotic beverage based on whey with addition of Mango and Almond pulp, in a ratio of 1:1, using *L. casei* as a probiotic micro-organism. They applied a hedonic scale preference test to assess the level of impact on smell, taste, and texture

with a group of 20 untrained judges. The scale used was structured with scores from 6 to 10; being 6 "I dislike much," 7 "I do not like," 8 "I do not like or dislike me," 9 "I like," and 10 "I like it a lot." The drink got a "I like" rating.

Legarová et al. [39] evaluated whey-based fermented beverages using the commercial yoghurt culture of *S. thermophilus* and *L. bulgaricus* to determine the effect of milk content on sensorial properties. They evaluated cold samples (4–6°C) using a panel of 15 people, an unstructured linear scale was designed. The samples prepared with whey and milk obtained the highest scores in all the descriptors, evidencing that a higher percentage of milk in the mixture, higher indexes of organoleptic quality in the drink.

Similarly, De Castro et al. [41] analyzed the sensorial acceptance of whey based fermented beverages with different concentrations of oligofructose (2 and 5%). They evaluated the samples after 72 h of preparation, using: (a) an untrained panel of 36 people, who were asked which drink they liked the most or did not like; and (b) an untrained panel of 50 people in one test of acceptability, using a structured hedonistic scale of 9 points (1—I do not like anything, 9—I like it very much) and a test of intention to buy using a scale of 5 points (1—definitely not going to buy it; 5—I will definitely buy it). Panelists preferred 2 and 5% oligofructose beverages compared to the control drink (without oligofructose), where acidity was an attribute mentioned by 41% of judges. Furthermore, in terms of acceptability, the average score for both drinks was over 7 points, evidencing that the variation in the oligofructose content added did not affect acceptance of the beverages.

On the other hand, Londoño et al. [18] carried out a sensorial evaluation of a fermented beverage made from fresh whey, using *L. casei* and commercial yoghurt culture of *L. bulgaricus* and *S. thermophilus*. They performed a drink acceptability test, using a panel of 80 people, considering a 9 points hedonic scale. The drink had a "like" rating. Pescuma et al. [38] evaluated the noble properties of whey-based functional beverages using LABs, which were made using commercial yoghurt culture of *L. bulgaricus* and *S. thermophilus* and a probiotic culture of *L. acidophilus*. They performed an acceptability test of the fermented drink, obtaining a grade of "I like."

Gallardo et al. [44] used a highly trained panel in the sensory evaluation of a whey-based fermented beverage with and without addition of hydrocolloids, with commercial yoghurt as an inoculum (0.02%).They performed the sensory evaluation under controlled conditions, following the ISO Standard 8589 (1988), using samples of 30 mL, in triplicate, on separate days. They used a 100 mm unstructured linear scale to measure the sensation of panelists leaving the sample for a maximum of 3 s before swallowing. According to the results, the addition of hydrocolloids affected the perception of the viscosity of fermented beverages substantially, evidencing the lack of a greasy sensation in the mouth.

In other studies, Hernández et al. [46] performed the sensory analysis on a reconstituted whey-based probiotic drink (7%) and pasteurized (80°C, 3 min) using three combinations (treatments) of *L. reuteri* and *B. bifidum* as inoculum T1: *L. reuteri* 1% and *B. bifidum* 0.5%, T2: *L. reuteri* 1% and *B. bifidum* 1%, T3: *L. reuteri* 2% and *B. bifidum* 0.5%. They used the triangle test and a panel of 10 people (8 women and 2 men) between 22 and 27 years old. They performed 12 tests per session, determining differences between treatments using a "d" value defined as

the difference between intensities for two products valued in standard deviation. The triangle test results showed sensorial differences between treatments T1 and T2; however, T3 was different from the first two. To confirm this difference, they performed a test in which 109 consumers participated, finding that 56% expressed differences by T3, 34.86% by T2 and 9.17% showed no preference, selecting T3 as final product, which after a descriptive test, was cataloged with "very good" organoleptic characteristics.

Following the same idea, Dalev et al. [47] conducted a qualitative sensory analysis of fermented whey and soy milk beverages to five probiotic beverages based on whey and soy milk: (1) unfermented soy milk (control drink), (2) fermented drink with equal volume of whey and soy milk. Starter culture: *B. breve* and *L. casei*, (3) fermented drink with equal volume of whey and soy milk. Starter culture: *B. infantis* and *S. thermophilus*, (4) fermented drink with equal volume of whey and soy milk. Starter culture: *B. animalis* and *L. plantarum*, and (5) fermented drink with equal volume of whey and soy milk. Starter culture: *B. breve*, *L. plantarum*, and *S. thermophilus.*

They performed the sensory evaluation using the Quantitative Description Analysis (QDA) method [61]. They selected descriptors or attributes to be evaluated and used a 10 cm unstructured linear scale, shown in monitors, converting the results on a numerical scale (from 0 to 10 units) expressing them in conventional units. They employed a panel of six people (four women and two men) trained according to the International Standards (ISO 1993), with at least 1 year of experience in descriptive tests of different foods, who received varied samples of 20 mL, each in triplicate. The results showed highly significant differences in attributes such as soy milk odor, cereal odor, fermented taste, strawberry odor, sweet taste, and after taste. They concluded that the addition of processed fruits helps to improve the characteristics of the beverages substantially, and therefore, the qualification as organoleptic quality. Soy milk has been used to prepare products such as yoghurt, but its poor organoleptic characteristics have been responsible for a very low acceptance by consumers.

2.5. Whey-based fermented beverages shelf-life

It is a fact that there are physico-chemical factors that can influence micro-organism survival in fermented beverages, being the most important acidity, temperature, oxygen concentration, type of inoculum and storage conditions. Theoretically, it is expected that for a reasonable time, the product will maintain the characteristics that define it as probiotic, so that quality can be guaranteed to the consumer. For this reason, many researches have evaluated the beverages shelf-life.

In this order of ideas, Fiorentini et al. [37] performed a viable lactic bacteria count in fermented beverages prepared from whey and soybean addition, after 7, 14, and 21 days storage under refrigeration at 7°C. They performed a selective count of *L. acidophilus* on modified MRS agar, with aerobic incubation and a count of *B. bifidum* on MRS agar modified with anaerobic incubation, both at 37°C for 72 h, finding that the number of alive micro-organisms in the fermented drink was 10^7 UFC/ml. Londoño et al. [18] evaluated the viability of the beverage based on fresh whey inoculated with *L. casei*. They performed counts on two types of agar,

MRS and M17, both at acidic pH (2.4) and neutral pH (7.2), finding that shelf-life of the product was 21 days. In addition, they did not observe marked variability in the results of the physico-chemical tests.

Similarly, Hernández et al. [46] performed a viable micro-organisms count to a beverage made from whey inoculated with *L. reuteri* 2% and *Bifidobacterium bifidum* 0.5%. Count was during storage at 4°C for 30 days. They counted *L. reuteri* on a modified medium of LBS agar, *B. bifidum* on MRS agar and total population of viable micro-organisms in MRS medium (pH = 5.5), finding that the beverage fulfilled the criterion of probiotic foods in an acceptable manner.

In other research, Sepúlveda et al. [20] carried out an evaluation of the physicochemical characteristics during the storage of a beverage prepared with whey with addition of Maracuyá pulp. As the days went by, a decrease in pH and an increase in acidity were observed, suggesting a shelf-life of no more than 21 days. Oliveira et al. [48] performed a microbiological analysis of four whey-based drinks inoculated with probiotic cultures at 1, 7, 14, 21, and 28 days of storage at 4°C. They used MRS-bile agar for counting *L. acidophilus*, MRS agar for *L. delbrueckii* ssp. *bulgaricus* and *L. rhamnosus* and agar for *S. thermophilus*. Although the probiotic count decreased during storage (28 days), they found that the beverages contained, on average, 5.3×10^6 CFU/mL of probiotics after 28 days of storage. In addition, they observed that, on average, pH remained at 4.5 after the first day of storage, showing a decrease between 0.14 and 0.32 units during the first week, decreasing slightly (less than 0.12 pH units) up to 28 days of storage, considering it stable.

Cóndor et al. [56] performed a microbiological evaluation of a whey-based beverage using immobilized *Kluyveromyces Marxianus* cells, during storage at room temperature and under refrigeration. They counted viable aerobic mesophiles, numbering of total coliforms and count of molds and yeasts, according to Peruvian Technical Standard 202.083 (1988). After storage viable aerobic mesophilic micro-organisms count and molds and yeasts (CFU/ml) were less than 10, while total coliforms (NMP/ml) were less than 3.0, indicating that the beverage was microbiologically acceptable. They also observed acidity and pH profile up to the fifth month of evaluation, both at room temperature (22°C) and under refrigeration (4°C).

3. Conclusions

Whey has been used for probiotic fermented beverages development significantly. It has excellent physico-chemical characteristics that make it become an excellent substrate, allowing probiotic bacteria growth in such a way that they reach high concentrations, achieving the probiotic effect and all of its health benefits. On the other hand, whey-based probiotic beverages have extraordinary organoleptic characteristic and they are widely accepted by consumers. An average shelf-life of these beverages is 21 days under refrigeration conditions, ensuring probiotics benefits during this period of time. Throughout this review, it is shown that probiotic consumption can be increased through the use of whey not only due to its excellent physico-chemical characteristics, but also due to its ability to develop beverages with high sensorial characteristics and due to its excellent acceptance.

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Chapter 8

Probiotics and Ruminant Health

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Additional information is available at the end of the chapter

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Abstract

Probiotics are viable microorganisms with beneficial health effects for humans and animals. They are formulated into many functional foods and animal feed. There is a growing research interest in the application and benefits of probiotics in ruminant production. Several recent studies have evaluated the potential of probiotics in animal nutrition and health. In this chapter, we have reviewed current research on the benefits of probiotics on gut microbial communities in ruminants and their impact on ruminant production, health and overall wellbeing.

Keywords: probiotic, ruminant health, gut microbiota, immune response

1. Introduction

The gastrointestinal tract of domestic ruminant animals mainly cattle, sheep and goat are inhabited by diverse and complex microbial communities including bacteria, protozoa, fungi, archaea and viruses. In the last three decades, there have been numerous research studies to characterize the gut and rumen microbiota population and understand their importance on ruminant nutrition and health. In dairy cows, the rumen, which is the main fermentation chamber contains different microbial communities; about 100 billion bacteria, protozoa, methanogens and other anaerobic fungi [1, 2]. The major microbial groups in the rumen include *Prevotella*, *Selenomonas*, *Streptococcus*, *Lactobacillus* and *Megasphaera.* The rumen is also predominately inhabited by fiber-degrading bacteria such as *Fibrobacter*, *Ruminococcus*, *Butyrivibrio* and *Bacteroides* [2]. These native microbial groups have important function in the digestion and fermentation of dietary polysaccharides by the host [3]. In addition, the rumen microbial population must be balance and healthy for efficient digestion of feed and impact animal health [4]. In ruminants, variation in the rumen microbiota between individual animals has

been reported. This variation is dependent on animal age, health status and environmental factors [5–8]. There is a growing research interest in the application of beneficial microbes/probiotics in ruminant production to help balance the gut microbiota, and as possible alternative to antibiotic use through improved gut health.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [9]. Probiotics are widely recognized as non-pathogenic microbes with health benefits [10]. The beneficial health effects of probiotics are related to their immunomodulatory activity in the gut by stimulating the secretion of immune modulators such as cytokines and IgA in intestinal mucosa [11]. In ruminants, probiotics are administered to target the rumen (main site of feed digestion) where they have an effect on rumen fermentation especially on feed digestibility and degradability and rumen microbiota [12]. Probiotic positively affect celluloysis and synthesis of microbial protein during digestion [13], and stabilizes rumen pH and lactate levels. In addition, probiotics are able to enhance nutrient absorption [14]. Direct-fed probiotic have been shown to reduce ruminal acidosis [15].

Lactic-acid bacteria strains such as *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Saccharomyces* and *Enterococcus* are commonly used as probiotics in functional foods and animal feed [16–20]. *Lactobacillus* and *Bifidobacterium* species have been shown to provide protection against enteric infection. These beneficial microbes consist of different species of microorganisms such as bacteria and yeast and they may be used as single or multi-strain. The multi-strain probiotics have a broad spectrum effect from the different strains against infections [21], and could increase their beneficial effects of probiotics due to their synergistic adhesion effect [22].

Probiotics are typically used to improve gastrointestinal health, reduce diarrhea, bloating and protect against infectious diseases [23]. Several researchers have reported the benefits of oral administration of probiotics to ruminants. Probiotics regulate and balance gut microbes, promote growth and development of animals, and improve the host resistance to diseases [24]. Recent studies suggest that utilization of probiotics as feed supplement for ruminants improves growth performance, production, and enhance health and overall wellbeing of the animals. Applications of probiotics have been shown to reduce the negative environmental impact such as methane emission associated with ruminant production. In this chapter, we have reviewed current research on the benefits of probiotics on gut microbial communities in ruminants and their impact on ruminant production, health and overall wellbeing.

1.1. Selection of probiotic strain

It is important to select the suitable strain of a microorganism for use as probiotic. The suitable potential probiotic strain is considered as an inhabitant of the host organism and has the ability to adhere and colonize the epithelial cells of the gut. Also, the potential probiotic microbe should be able to grow and survive in the host [25]. Microbial strains used as probiotics are required not to affect the indigenous gut microbiota population of the host. Other important requirement for the potential probiotic strain is to be able to adapt to the environment of the gut and locate a suitable niche in the rumen (such as epithelium, fluid or feed), and exerts positive effects on the host [8]. Other Safety criteria and characteristic of probiotics to consider

include, non-pathogenic, resistance to gastric juice and bile, anatgonize pathogenic bacteria, genetically stable, and exhibit stable qualities during processing, storage and delivery, viable at high populations [16]. In the USA, there are regulatory considerations by the Food and Drug Authority for safety evaluation of microorganisms used as probiotic. The specific microorganism should have "Generally Regarded As Safe" (GRAS) status [26].

1.2. Different types of probiotic microorganisms

There are different microbial species used as probiotics in ruminants which include bacteria, yeast, etc. **Table 1** presents a list of microorganism targets commonly used as probiotics in ruminants feeds and this includes bacteria species belonging to the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and yeast strains such as *Saccharomyces cerevisiae* and *Kluyveromyces* [29]. The most common commercial probiotics products for ruminants consist of live yeast (*Saccharomyces cerevisiae*). Although, majority of these strains are nonpathogenic and safe, others especially *Bacillus cereus* produces enterotoxins which may not be safe [29]. The use of yeast and fungal probiotics are more effective in adult ruminants, whereas probiotic containing bacteria species have high efficacy in pre-ruminant

***Lactobacillus* species**	***Bifidobacterium* species**	***Enterococcus* species**	**Other species**
L. reuteri	*B. longum*	*E. faecalis*	*Sporolactobacillus inulinus*
L. paracasei	*B. breve*	*E. faecium*	*Bacillus cereus*
L. brevis	*B. infantis*		*Saccharomyces boulardii*
L. lactis	*B. adolescentis*		*Streptococcus Salivarius* subsp. *thermophilus*
L. johnsonii	*B. lactis*		*Clostridium botyricum*
L. crispatus	*B. animalis*		*Escherichia coli*
L. fermentum	*B. bifidum*		*Lactococcus lactis* subsp. *lactis*
L. amylovorus			*Lactococcus lactis* subsp. *cremoriss*
L. delbrueckii subsp. *bulgaricus*			*Pediococcus acidilactici*
L. rhamnosus			*Propionibacterium freudenreichii*
L. helveticus			*Leuconostoc mesenteroides* subsp. *dextranicum*
L. acidophilus			
			Aspergillus oryzae
L. gallinarum			*Aspergillus niger*
L. plantarum			*Kluyveromyces fragilis*
L. casei			*Kluyveromyces marxianus*
L. salivarius			*Saccharomyces pastorianus*

Table 1. Common probiotic microorganisms use for ruminant (Adapted from [16, 17, 27, 28]).

calves. In pre-ruminants since their rumen is not yet developed, probiotic species administered targets the small intestine, help balance the gut microbiota, and reduce pathogen colonization of the host [30].

Prebiotic are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth/or activity of one or a limited number of bacteria in the colon" [31]. Prebiotics are commonly dietary fiber and have effect on both the upper and lower GI tract. In the upper GI tract, prebiotics are able to withstand digestion, delay gastric removal, decrease glucose absorption and stimulate the release of intestinal hormonal peptides. The main prebiotics used in animal diet are carbohydrates and oligosaccharide. Non-digestible oligosaccharides used include oligofructose, inulin, lactulose, galactooligosaccharide, transgalactooligosaccharide [16, 32].

Synbiotics on the other hand are products that contain a mixture of probiotic and prebiotics. The host benefit from the synergistic effect of probiotic and prebiotic. Results from studies done have demonstrated the promising effect of synbiotics in reducing the numbers of food borne pathogens [33].

1.3. Administration of probiotics

There are different route of administration of probiotics. These sites include the oral cavity, intestines, vagina and the skin [34]. In ruminants, probiotics are usually administered orally [35–43]. A study by Deng et al. [44, 45] utilized intravaginal infusion as mode of administering probiotics (containing a lactic acid bacteria mixture) to periparturient cows.

2. Probiotics and ruminant growth and production performance

2.1. Effect of probiotic on growth performance

Utilization of probiotics (either dry or live) as natural feed additives have been shown to favorably improve animal performance and welfare, via modulation of gut microbial community which is essential in ensuring host homeostasis [46]. Probiotic have positive effect on growth rate and production performance of animals when administered as single or multi-strain feed supplement (**Table 2**). Oral administration of probiotic has been shown to improve feed intake, daily weight gain and overall weight gain in sheep, goats, and cattle [38–43, 47, 49, 51, 52]. The population of beneficial microbes such as *Lactobacillus* and *Bifidobacteria* are low in neonatal calves, but studies have shown that supplementation with probiotics containing these microbes increases their growth [55]. In dairy cows, probiotic composed of live yeast increased food intake, improved feed efficiency, improved average daily gain and overall total weight. Additionally, probiotic increased milk yield and quality [51, 52].

In small ruminants such as goats and sheep, treatment with commercial probiotic improved average daily gain [35]. Gyenai et al. [36], Ekwemalor et al. [37] and Ekwemalor et al. [41], reported contrary results where there was no effect of probiotics on body weight. These different

Probiotics type	Ruminant	Performance effect	References
Lactobacillus casei ssp casei	Calves	Weight gain	[47]
Lactate-utilizing/or lactate-producing bacteria	Cattle	Improve feed efficiency, increase in daily gain (2.5%)	[48]
Calf-specific probiotic (six *Lactobacillus* species)	Veal calves	Reduced diarrhea	[49]
		Decreased fecal coliform counts	
Live yeast	Beef cattle	Improve average daily gain, final weight, feed intake, feed to gain ratio	[50]
Yeast	Dairy cows	Increased milk yield and quality	[51, 52]
		Increase feed efficiency	
		Reduced ruminal acidosis	
Lactobacillus casei Zhang and *Lactobacillus plantarum* P-8	Dairy cows	Improve quality and quantity of milk production	[24]
FasTrack Microbial pack (*Lactobacillus acidophilus, Saccharomyces cerevisiae, Enterococcus faecium, Aspergillus oryzae, fructooligosaccharide*, active dry yeast culture	Dairy cows	Improve body weight	[42, 43]
Lactobacillus acidophilus, Saccharomyces cerevisiae, S. boulardii, Propionibacterium freudenreichii	Lactating cows	Increased milk production	[53]
Bacillus licheniformis and *Bacillus subtilis*	Sheep	Reduced lamb mortality rate	[54]
		Increased average daily milk yield per ewe	
		Improved fat and protein content of milk	
Probiotic mixture (*Bifidobacteriumlongum*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Lactobacillus rhamnosus*)	Goat	No effect on body weight, PCV, White blood cells differential count	[36]
Mushroom-based probiotic (Coriolus versicolor)	Goats	No effect on body weight, PCV, White blood cells differential count	[37]
Commercial probiotic	Meat Goats	Improved average daily gain	[35]
Multi-strain Probiotic (*Lactobacillus acidophilus, Saccharomyces cerevisiae, Enterococcus faecium, Aspergillus oryzae, fructooligosaccharide*, active dry yeast culture)	Goats	Improved Packed cell volume and FAMACHA scores	[41]

Table 2. Summary of benefits of probiotics on growth and production performance of ruminants.

observations reported may be due to difference in the probiotic composition used, amount use, specific activity of the probiotic strains and variation in the breeds of goats used in their individual studies. This because studies have shown that different probiotic strains may have different effects depending on their capabilities and enzymatic activities different host species [34]. In the study by Gyenai et al. [36] Spanish Boer kid-goats were drenched with a probiotic mixture consisting of *Bifidobacterium longum*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Lactobacillus rhamnosus*. The commercial probiotic used by Ekwemalor et al. [41] composed of *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Aspergillus oryzae*, *fructooligosaccharide*, active dry yeast culture. Whitley et al. [35] used a commercial probiotic containing active dry yeast and lactic acid-producing bacteria, including *Lactobacillus acidophilus* and *Enterococcus faecium*. In addition, Whitley et al. [35] tested the probiotic on Boer crossbred meat goats (50–75% Boer of genetic background) however, Spanish Boer goats were used in the studies by Gyenai et al. [36] and Ekwemalor et al. [41]. Furthermore, two indicators of anemic condition in goats, Packed cell volume and FAMACHA have been reported to be affected by probiotic treatments [41].

2.2. Effects of probiotics in milk

Use of probiotics as feed supplements for ruminants have beneficial influence on milk production, milk quality and functional components such as protein and fat content [24, 54]. Studies have shown that probiotic dairy products are safe for large-scale consumption [56]. A study conducted by Yu et al. [57] showed that dairy cows treated with probiotic species *Aspergillus oryzae* ad *Saccharomyces cerevisiae* increased milk production and milk proteins. Also Sun et al. [58] and Qiao et al. [59] found that probiotics containing *Bacillus subtilis* improved the milk yield and rumen fermentation of dairy cows. Stein et al. [51] and Stella et al. [60] reported that probiotics improved the feed utilization rate, the milk yield and component profiles, and increase the dry matter intake in dairy cow. Xu et al. [24], also reported that probiotic application could reduce udder inflammation and increase milk yield while suppressing somatic cell count. Sun et al. [58] and Lehloenya et al. [61] reported that probiotic administration to dairy cows increased the milk production and simultaneously improved the milk fat, protein and lactose yield, accompanied by a decrease in milk somatic cell count. These positive effects of probiotics on milk production and milk quality characteristics are attributed to the subsequent effects of probiotics on the number of cellulolytic and fiber-degrading bacteria as well as changes in the volatile fatty acid in the rumen [54].

3. Molecular mechanism of action of probiotics

The mode of action of probiotics in the host organism include: regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function, expression of bacteriocins enzymatic activity inducing absorption and nutrition, immunomodulatory effects, inhibition of procarcinogenic enzymes and interference with the ability of pathogens to colonize and infect the mucosa [62]. In ruminants, the mechanism of probiotics metabolism is dependent on the strain of microorganism used. Probiotic bacteria can serve to decrease the severity of infection via a number of mechanisms including competition for receptors and

nutrients, and/or the synthesis of organic acids and bacteriocins that create an environment unfavorable for pathogen development [49, 63–66].

4. Probiotics and immunity

The beneficial health effect of probiotics have been partly attributed to the ability of probiotic bacteria to modulate the immune system, increasing both innate and adaptive immune response [67, 68]. Research evidence obtained from various in vivo and in vitro studies have demonstrated that probiotic promote gut health via stimulation of the innate immune response [69]. Different probiotic bacteria including Lactobacillus casei, *Lactobacillus casei* strain Shirota, *Streptococcus thermophilus*, *Lactobacillus fermentum* and yeast have been tested to elicit an immune response [67, 70]. Oral administration of *Lactobacillus casei* activated immune cells of the innate immune response, and increased the expression of innate immune receptor, TLR2 [70]. In a similar study in mice, administration of *Lactobacillus casei* strain Shirota (LcS) enhanced innate immune response by stimulating or inhibiting the production of TH1/TH2 cytokines [67, 71]. Ghadimi et al. [71] reported that probiotic enhanced secretion of IFN-γ (a TH1 cytokine) and inhibited the stimulation of TH2 cytokines such as IL4 and IL5. Research findings by Yan and Polk [72] showed that immunomodulatory effect of a probiotic (*Lactobacillus rhamnosus* GG) in preventing cytokine-induced apoptosis in intestinal epithelial cells. Results from their study indicated that the probiotic inhibited activation of the p38/mitogen activated protein kinase which is a pro apoptotic kinase induced by cytokines TNF, IL-1α, or IFN-γ. Furthermore, the *Lactobacillus rhamnosus* GG probiotic activated Akt/protein kinase B (anti-apoptotic) in colon cells from mice and humans. In an *in vitro* study, a multiple probiotic formulation has been demonstrated to activate NF-κB and stimulate production of TNF-α in epithelial [69]. Studies done in vivo and ex vivo have demonstrated the effect of probiotics treatment on the inflammasome [73]. The inflammasome found in various immune cells (macrophages and dendritic cells) and intestinal epithelial cells consists of cytosolic proteins such as NOD-like receptors, apoptosis-associated speck-like protein containing a CARD domain and caspase-1 (the serine protease). Activation of inflammasome receptors further leads to activation of caspase-1 and Interleukin (IL)-1β and IL-18. Activation and secretion and of the inflammatory cytokines Interleukin (IL)-1β and IL-18 stimulates and enhance the antimicrobial effect of immune cells against intracellular pathogens infection and also activates cell death of inflammasome-activated cells [74–76]. Studies have reported a potential role of the inflammasomes in the development of chronic intestinal inflammation [73, 76]. In ruminants such as bovine, the probiotic *Lactobacillus rhamnosus* GR-1 have been shown to amend *E. coli* induced inflammation in primary bovine mammary epithelial cells. Findings from their study showed that probiotic pretreatment impaired the activation ASC-independent NLRP3 inflammasome, and decreased protein expression of NLRP3 (NOD -like receptor family member pyrin domain-containing protein 3) and caspase 1 induced by *E. coli* [77].

The molecular impact of oral probiotic supplementation on systemic expression of genes associated with innate immune response in blood have been reported for ruminant species; cows [38, 42, 43], and goats [41] as shown in **Table 3**. Probiotics have been reported to activate pathways immunity and homeostasis including Toll-like receptor pathway, Wnt signaling pathway,

Innate immune response parameters	Genes	Ruminant type	References
Toll-like receptors	TLR2	Goats	[37]
	TLR8	Dairy cow	[38, 43]
	TLR6		
	TLR7		
Cytokines	IL4	Goats	[37]
	IL6	Dairy cow	[43]
	IL1B		
	IFNB1		
	CCL2		
	CCL3		
	CCL19		
	IL16		
	IL10RA		
Chemokines	CXCR2	Cattle	[43]
	CXCR1		
	CCL2		
	CXCL8		
Th1 marker	STAT4	Goats	[37]
	CXCR3		
Wnt signaling	WNT8A	Dairy cow	[37, 42, 43]
	WNT5A	Goats	
	WNT10B		
	KREMENS		
	DVL1		
	PRICKLE3		

Table 3. Effect of probiotics on innate immune response gene expression in ruminants.

innate and adaptive immune response pathway [38, 41–43]. A study by Ekwemalor et al. [41] showed that oral probiotic administration may exhibit systemic effect in goat blood, by modulating the expression of genes associated with immunity and homeostasis. In goats, probiotic treatments induced the expression of 32 innate immunity genes and 48 genes in the Wnt signaling pathway. Furthermore, treatment of goats with a mushroom based probiotic in an vivo study trial resulted in serum increase in pro-inflammatory cytokines such as interferon production regulator (IFNr), Rantes and Granulocyte-Colony Stimulating Factor (GCSF). But the level of granulocyte macrophage colony stimulating factor (GM-CSF) reduced [37].

In dairy cows, oral probiotic supplementation had systemic effect on differential global gene expression. Probiotic treatment targeted 87 bovine pathways including Wnt signaling pathway, inflammatory response pathway, toll-like receptor signaling pathway, prostaglandin synthesis and regulation pathway and B cell receptor signaling pathway. Probiotic treatment modulated the expression of genes associated with innate immunity and homeostasis such as receptors TLR2, TLR6, TLR7, TLR8; cytokines, IL16, IL6, IL10RA; Wnt signaling genes Wnt8A, Wnt5A, Wnt10B, Kremens; and transcription regulators MAP4K3 and MAP3K8 [38, 42, 43].

5. Application of probiotic in ruminant

5.1. Probiotics and cattle

Probiotic have been widely used in cattle production for both dairy and beef cows at all developmental stages and growth. Studies have shown the beneficial effect of direct-fed microbials or probiotic bacteria including *Lactobacillus* and *Bifidobacteria* on growth, production performance (milk production, milk functional components and milk composition) and immune response of dairy cows, beef cattle, neonatal calves, and periparturient cows [38, 43, 51, 52, 55]. Furthermore, probiotic supplementation showed potential effect to decrease ruminal acidosis in feedlot cattle and dairy cows, and also improved immune response in stressed calved [48]. In dairy cows, probiotic increased food intake, improved feed efficiency, and improved average daily gain. Additionally, probiotic increased milk yield and quality [51, 52]. There are limited studies on the effect of probiotics on beef cattle compared to the research conducted on dairy cows. However, the use of probiotic yeasts to improve beef production has been variable, possibly due to the diet composition, strain of yeast or yeast viability [78].

Studies by Krehbiel et al. [48] have shown that probiotics are effective decreasing fecal shedding of Escherichia coli O157:H7 in infected calves. Research findings reported by Sherman et al. [79], demonstrated that treatment of intestinal cells with lactic acid producing bacteria reduced epithelial injury due to *E. coli* O157:H7 and *E. coli* O127:H6 exposure. Therefore, probiotic is used as one of the many strategies to reduce shedding of *E. coli* o157:H7 and non-O157:H7 in ruminants [80].

5.2. Probiotics and goats

Utilizing probiotics as functional food supplement have been encouraged in goat production [81]. Various commercial probiotic products consisting of either single strains or mixture of strains such as *Lactobacillus reuteri* DDL 19, *Lactobacillus alimentarius* DDL 48, *Enterococcus faecium* DDE 39 and *Bifidobacterium bifidum* DDBA have been tested in goats [35, 81]. Probiotic administration significantly increased body weight, and modified microflora by increasing the number of lactic acid bacteria and *Bifidobacteria* of goats. In addition, probiotic treatment reduced fecal mutagenicity by 60%, which is an indication of the protective influence of probiotics in goats [81]. Whitley et al. [35], observed no effect of probiotics on growth performance, diet digestibility, carcass traits, or fecal microbial populations in meat goats, although

an effect on the average daily gain was observed. Research findings by Gyenai et al. [36] supports the use of probiotics in goats to enhance microbial retention in the rumen.

There is an increasing market demand for nonfat goat milk and milk products such as yoghurt containing probiotics. A nonfat yoghurt has been developed from goat milk and is enriched with probiotic strains *Lactobacillus acidophilus* and *Bifidobacterium spp.* [82]. Other probiotic microbes have been used to develop different types of fermented drinking milk form goat milk. *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB-12 novel putative probiotic *Propionibacterium jensenii* 702 co-culturing in goat milk affected their viability and physico-chemical properties of the milk [83]. In a similar study, goat milk fermented with *Lactobacillus fermentum* ME-3 and tested in healthy human subject reduced peroxidized lipoproteins levels, decreased 8-isoprostanes, improved total antioxidant activity and demonstrated an anti-atherogenic effects. The population and activity of lactic acid bacteria in milk was affected after fermentation with *Lactobacillus fermentum* ME-3 [84].

5.3. Probiotics for sheep

In sheep production, probiotics have been applied to improve feed digestion and gut health. Two probiotics, *Saccharomyces cerevisiae* and *Aspergillus oryzae tested in* sheep had no effect on Nitrogen digestibility *and* net microbial protein flow in the duodenum [85]. In another study, a probiotic mixture containing *Bacillus licheniformis* and *Bacillus subtilis* administered in ewes at late pregnancy and lactation reduced mortality in young lambs. In addition, probiotic treatment increased daily milk yield per ewe and fat and protein content of milk were also increased [54]. In a study by Rigobelo et al. [86], administration of a probiotic mixture containing *Lactobacillus acidophilus, Lactobacillus helveticus, Lactobacillus bulgaricus, Lactobacillus lactis, Streptococcus thermophilus* and *Enterococcus faecium* to sheep infected with a non-O157 Shiga toxin-producing *Escherichia coli* (a foodborne pathogen of humans) reduced the fecal shedding of the pathogen. Probiotic treatment in sheep has beneficial effect on rumen methanogenesis, energy retention and Nitrogen utilization. In particular adding yeast culture and β1–4 galacto-oligosaccharides decreased methane emission in sheep [87] Propionibacteria- and lactobacilli-based probiotics were tested in sheep modified the bacterial population have been suggested to be useful to reduce the incidence of butyric and propionic subacute ruminal acidosis in sheep [88].

A study conducted in sheep showed that probiotic microorganisms are been used to improve food safety for consumers. Delcenserie et al. [89], found in their study that the presence of *Bifidobacteria choerinum* may be used as an indicator of fecal contamination of mutton. The study findings suggest that detection and identification of Bifidobacteria correlated with *E. coli* numbers can be used to improve hygienic quality during mutton processing.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

Probiotics, an Alternative Measure to Chemotherapy in Fish Production

Olumuyiwa Ayodeji Akanmu

Additional information is available at the end of the chapter

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Abstract

The use of chemotherapy in treating and enhancing the growth of fish has been widely criticized due to its negative environmental consequence. Hence, the use of probiotics which are bio-friendly seems to be a promising alternative. Therefore, the importance of probiotics in fish production was critically reviewed in line with their growth rate, disease treatment, and immune boosting. It was, however, realized that probiotics such as *Lactobacillus fermentum* and *Saccharomyces cerevisiae* cultured from maize slurry and palm wine, respectively, could serve as good probiotics, which could enhance faster growth rate and wound-healing rate. Probiotics are, therefore, recommended to the fish farmers so as to increase the profitability of the aquaculture business.

Keywords: *Lactobacillus fermentum*, *Saccharomyces cerevisiae*, probiotics

1. Introduction

Aquaculture is an ancient occupation of man in which its fast growth due to rapid development has given birth to modern equipment and technology leading to its intensification and commercialization. This development has placed disease problems on the threatening side, making it to be a major constraint to the culture of many aquatic species and consequential impediment on economic and social development in many countries [1]. Owing to the artificial conditions posed by intensive rearing, farmed fish is more susceptible to disease agents than fish in natural aquatic environments [2]. Fish diseases constitute the major limiting factor in aquaculture production since the disease causative agents thrive well in water. Various types of bacterial diseases in fish have been encountered in fresh water fishes across the globe [3]. Jakhar et al. [4] reported that bacterial pathogens cause heavy mortality in both cultured and wild fish/shell species over the world.

However, prevention and control of diseases have led to a substantial increase in the use of broad-spectrum chemotherapeutics, which has been reported to cause development of resistant bacteria, reduction in yield, and introduction of potential hazard to public health, the environment, and killing of the microbial flora in the digestive tracts, which is beneficial to the fish [5]. The development of resistant bacterial genes as a result of exposure to antimicrobial agents has not only made the drugs applied useless, but has also made the animals treated with it not safe for human consumption; therefore, it turned the treatment exercise to a wasteful process, which eventually makes this to be a major disadvantage of using synthetic antibiotics in aquaculture [6]. The success of modern aquaculture among others hinges on the use of biological control agents for diseases, and this depends on the fact of microbial antagonism [3] and triggering immune response to disease challenge.

Generally, the immune system of aquatic organisms is affected by periodic and unexpected changes in their environment. Adverse environmental situations may acutely or chronically, stress the fish, altering some of their biochemical parameters and suppressing their innate and adaptive immune responses [7]. This triggers nonspecific defense mechanisms, which plays important role at all stages of infection. Fish, particularly, depends more heavily on these nonspecific defense mechanisms than mammals. Therefore, there has been an increasing interest in boosting the nonspecific immune system of fish for the treatment and prophylactic measure against disease in the last decade using biological and eco-friendly approach [8].

Probiotics are microbial dietary adjuvant that beneficially affect the host physiology by modulating mucosal and systemic immunity, as well as improve nutritional and microbial balance in the intestinal tract [9]. These biological agents have been utilized for disease control, supplements to improve growth, and in some cases as a means of replacing antimicrobial compounds in aquaculture. Probiotics have proven to inhibit the growth of pathogens through production of antagonistic compounds, competition for attachment sites, nutrients, and alterations of enzymatic activity of pathogens, immune-stimulatory functions, and nutritional benefits such as improvement in digestibility and utilization in feed [10]. Hence, the concept of utilizing probiotics in animal feed, particularly, poultry and fish, is fast gaining acceptance [11]. The objective of prevention and control of disease can be achieved by the use of probiotics. Probiotics are characterized by their ability to adhere and colonize the gastro intestinal tract (GIT) of the hosts and able to replicate to high numbers. These organisms must be able to produce antimicrobial substances and withstand the acidic environment of the GIT of the host animals. Probiotics are known to play an important role in developing innate immunity among the fishes; therefore, help them to fight against any pathogenic bacteria as well as against environmental stressors [11].

Probiotics can be introduced into culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms. The use of probiotics will prove a new eco-friendly alternative measure for sustainable aquaculture. A wide range of gram positive bacteria have been evaluated as probiotics. This includes *Aspergillus oryzae, Lactobacillus, Bacillus, Micrococcus, Carnobacterium, Enterococcus, Streptococcus, and Saccharomyces species*. The products of probiotics could be administered through water or incorporated in feed, either singly or in combination [11]. Administration of the probiotics proved harmless to the host as well as human being; it also results in improved resistance to infectious diseases in the hosts. However, the dimensions of the effects of probiotics have to be assessed for different fish species. Probiotics could be prepared in different types which

include: nonviable, which are dried probiotics; freeze-dried, which are probiotics that thrive well at freezing point; fermentation probiotics, which are produced through fermentation; and viable probiotics, which are living probiotics with guaranteed shelf life [12]. Probiotics have been demonstrated to have potentials for enhancing fish immunity [13], growth [14], wound healing [15], and are eco-friendly [16]. A successful probiotic is expected to be antagonistic to pathogens, by producing antimicrobial substances, which are harmful to the pathogens. In addition, the probiotics should have the capacity to colonize the fish by adhesion and produce important substances like vitamins, which has beneficial effect on the host, in the form of growth promotion or protecting the fish against bacterial pathogens [17].

Probiotics such as *Lactobacillus, Saccharomyces species* and their combinations have been found useful in aquaculture production [15, 18]. The administration of diets fortified with these probiotics have improved growth in *Oreochromis niloticus* [14], increased immunity in *Cyprinus carpio* [19], improved wound healing in *Clarias gariepinus* [20], and in *Heterobranchus bidorsalis* [15]. It would be of interest to understand the applicability of this bio-technique in advancing the fate of aquaculture and food security. Maintenance of hygiene and especially, chemotherapeutics are widely used as interventions on control of diseases of aquatic animals. However, intensive use of chemicals had contributed to the development of resistant strains of pathogens. Hence, there is the need for natural preventives for improving resistance in fisheries and aquaculture. Meanwhile, a large percentage of culture systems still depend mostly on the use of chemotherapeutic agents in treating and controlling the widespread of these diseases. The abuse of chemotherapeutics in fish farming has led to development of drug-resistant bacteria and multiple antibiotic-resistant in the aquaculture industry [21]. This approach has sometimes resulted in the spread of epizootic diseases and severe economic losses. Moreover, chemotherapy may kill or inhibit the normal micro flora in the digestive tract, which is beneficial to fish [22]. Therefore, there is an urgent need to develop alternative approach to the indiscriminate use of antibiotics in fish production.

The principal objectives of the food security through aquaculture can only be achieved in the face of increase in growth and survival, feed efficiency, and disease resistance of culturable fish species, which reflects positively on production costs. The use of probiotics, which control pathogens through a variety of mechanisms that targets these attributes are viewed as an alternative to the use of antimicrobial agents [23] but the potentials of this technique have to be tested in many indigenous culturable fish species. With increasing demand for eco-friendly aquaculture, the use of probiotics in aquaculture is now widely accepted [24]. Positive effects of applying certain beneficial bacteria in aquaculture have also been well documented [25].

2. Standards considered in selecting microorganisms as probiotics

For a microorganism to be considered as a good probiotics candidate, it should be able to exhibit these properties: antagonistic properties through the production of antimicrobial materials such as hydrogen peroxide [26] or siderophores [27]. They should be able to colonize other microorganisms in the fish organ through adhesion [17]. The microorganisms are expected to be viable for long period of time under storage [28]. Adhesion is one of the most

important criteria for probiotic bacteria because it is considered a pre-requisite for colonization [29]. Probiotic microorganisms will of course have to be nonpathogenic and nontoxic in order to avoid undesirable side effects when administered to fish. Tests of antagonisms, which include studies of adhesion and in-vitro challenged tests, challenged experiments in which fish treated with friendly bacteria are subjected to pathogens in order to evaluate the efficacy of the probiotics by using survival rate as an indicator are important considerable factors in selecting probiotics [30]. The interest of the probiotic use is centered on terrestrial organisms and the term probiotic inevitably is referred to gram positive bacteria associated with the genus *Lactobacillus species.* Panigrahi et al. [28] submission, however, requires some considerations to humans and terrestrial animals. It could be assumed in aquaculture that the intestinal microbiota does not exist as an entity by itself but there is a constant interaction with the environment and the host functions [31]. The bacteria in the aquatic medium could either be ingested with the feed or when the host drinks water. Terrestrial animals (mammals) inherit an important part of the initially colonizing bacteria through contact with the mother, while aquatic species usually spawn eggs in water, without further contact with their parents. This allows the ambient bacteria to colonize intestinal tract, gills, or skin of newly born animals/larvae, which have not fully developed.

3. Test for pathogenicity of the selected strains

Microorganisms considered as probiotic candidates should be scrutinized for pathogenicity on the host animals by challenging the target animals with the probiotic microorganisms. The challenged organisms could be administered to the target species through injection, immersion, or addition into the feed. The test of pathogenicity could either be carried out in-vitro or in-vivo.

a. **In-vitro antagonism tests:** Common way to screen the candidate probiotics is to perform in-vitro antagonism tests in which the pathogens are exposed to the candidate probiotics or their extracellular products in liquids [32] or solid medium. Depending on the extract arrangement of the tests, candidate probiotics can be selected based on the competition for nutrients [32]. The pre-selection of probiotics candidate based on these in-vitro antagonism tests has often led to the finding of effective probiotics [26].

b. **In-vivo antagonism tests:** Pathogenicity effects of microorganisms considered as probiotics could also be tested in-vivo to determine the safety level of the tested probiotic candidate.

4. The probiotics characteristics of Lactic acid bacteria and Yeast

Lactic acid bacteria are potential probiotic candidates in aquaculture and are also known to be a normal inhabitant in the intestine of healthy fish [14]. Most lactic acid bacteria are harmless, while some strains have been reported to have beneficial effects on fish health and are antagonistic to pathogens [32]. Strains of lactic acid bacteria are the most common microbes employed as probiotics. Most probiotic strains belong to the genus *Lactobacillus*. *Lactobacillus species* have

the ability to degrade organic materials, reduce ammonia, and inhibit the growth of pathogens by outcompeting them [33]. Lactic acid bacteria are a heterogeneous group of bacteria that are generally considered safe for use in food and food products. Lactic acid bacteria have been used for lactic acid fermentation of sorghum-or maize-based cereals used as infant weaning foods, for example, Pap (*ogi*) prepared from maize slurry [34]. Lactic acid bacteria are spherical, cocci, coccobacilli, or rods and divide in one plane only with the exception of *Pediococcus species* [35]. Lactic acid bacteria have no strict taxonomic significance although they have been shown by serological techniques and 16S ribosomal RNA cataloging to be phylogenetically related. They share a number of common features as earlier stated. Most of these organisms are aero-tolerant anaerobes, which lack cytochromes and porphyrins. The lack of these two components in their systems explains why they are negative to catalase and oxidase tests [36]. The antibacterial effect of lactic acid bacteria (LAB) is therefore ascribed to its tendency to produce antibiotics-like substances (bacteriocins) such as *Acidophilin, Lactolin,* and *Lactocidin*.

Saccharomyces cerevisiae is budding yeast species commonly used in baking and brewing, owing to its fermenting property or ability. The cells are ovoid in shape, 5–10 μm in diameter. It reproduces by a division process known as budding. All strains of *S. cerevisiae* can grow aerobically on glucose, maltose, and trehalose but cannot grow on lactose and cellobiose. It is a single-celled organism, which can easily be cultured with short generation time of about 1.5–2 hours doubling time at a temperature of 30°C. It contains various immune-stimulating compounds such as β-glucans, nucleic acid, and mannan oligosaccharides, which have been reported to enhance immune response and growth of various fish species [37]. Mesalhy et al. [38] recorded higher growth rate in the study carried out using probiotic-supplemented diets on *Oreochromis niloticus* than those kept on basal diet. It was concluded that addition of *Bacillus subtilis* and *S. cerevisiae* enhanced the growth performance, feed utilization, and mitigated the effects of population density, which is the main growth-inhibiting factor in intensive aquaculture systems. The best food conversion rate (FCR) values were recorded in probiotic-supplemented diets, and it was concluded that the probiotic used improved feed utilization, which practically showed that the probiotic used can reduce the amount of feed necessary for animal growth and thus, reduce the cost of production [37].

Saccharomyces, Clostridium, Bacillus, Enterococcus, Lactobacillus, Shewanella, Leuconostoc, Lactococcus, Carnobacterium, and Aeromonas species are the commonly used probiotics in fish culture practices [11]. These probiotics have been reported to produce beneficial results to the host organisms. *Bacillus* species increased survival and production of channel catfish (*Ictalurus punctatus*), improved growth and immunity of Nile tilapia (*Oreochromis niloticus*) was achieved through feeding of diet containing *Bacillus subtilis* and *Rhodopseudomonas*, and rainbow trout (*Oncorhynchus mykiss*) was protected against *Vibrio anguillarum* by *Pseudomonas fluorescens* [9]. Generally, probiotics have demonstrated the ability to increase fish growth by enhancing the feed conversion efficiency, as well as confer protection against harmful bacteria by competitive exclusion, production of organic acids (formic acid, acetic acid, and lactic acid), hydrogen peroxide, and several other compounds [37]. They can also effectively trigger the fish immune system [37].

Abdul El-Halim et al. [39] discovered that the addition of living yeast in diet improved the performance of *Oreochromis niloticus*. Scholz et al. [40] also reported improved growth and survival of sea bass fry with *S. cerevisiae* and attributed this to adherence ability of *S. cerevisiae* cells to the gut

and secretion of amylase enzymes, which increased digestibility of the diet. The probiotics used by Marzouk et al. [41] enhanced the growth performance of *Oreochromis niloticus* and suppressed the activity of the pathogenic bacteria in the intestine of the tested fish. The disease outbreak was reportedly prevented in fish with the use of *S. cerevisiae* and *Bacillus subtilis* as probiotics. This could have been possible with the ability of these microorganisms to attach and colonize the intestinal walls of the host animals, which eventually prevent other bacterial from getting access to the intestinal walls [41]. Li et al. [42] described positively influenced growth performance of brewer's yeast (*S. cerevisiae*) and feed efficiency of hybrid striped bass (*Morone chrysops xm saxatilis)* and resistance to *Streptococcus iniae* infection. In addition, results of immune response assays illustrated that brewer's yeast can be administered for relatively long periods without causing immune-suppression.

Lactobacillus fermentum and S. cerevisiae have also been reported to improve the growth performance and health status of fish species, *Oreochromis niloticus* [37] and *Mystus montanus* [3]. Various studies have been carried out using some bacteria strains as probiotics on fish species such as *Clarias gariepinus* and *Tilapia species,* but there is little or no information on the use of bacteria strains and yeast species as probiotics on indigenous species such as *H. bidorsalis.* Furthermore, information on the immune response of these probiotics on this fish species is not available [43]. *Lactobacillus fermentum* is a common bacteria strain, which has been used on different fish species. The wide occurrence and high antagonistic effects to the pathogens of the *L. fermentum and the S. cerevisiae* made them a good potential in testing for their probiotic ability and immune response. A beneficial effect by application of certain beneficial bacteria in human, pig, cattle, and poultry nutrition has been well documented by Jong [44]. However, the use of such probiotics in aquaculture is a relatively new concept [45]. Zhou et al. [17] reported the use of beneficial bacteria (probiotics) to displace pathogens by competitive processes being used in animal industry as a better remedy than administering antibiotics. This phenomenon is now gaining acceptance for the control of pathogens in aquaculture.

5. Importance of probiotics in aquaculture

Probiotics have been found beneficial in various ways such as:

- Providing additional nutrients thereby reducing feed costs.
- Maintaining desired conditions within the culture environment.
- Eliminating the stressors like NH_3, NO_2, and NO_3.
- Stabilizing and controlling the microbial populations.
- Maintaining stable water quality parameters.
- Preventing bacterial and viral infections.
- Improving feed and make it to be more attractive.
- Supporting growth through production of vitamins, minerals, nucleic acids, and by stimulation of beneficial gut flora.

- Improving feed conversion rate and survival rate of aquatic species.
- Reducing the use of chemotherapy.

The benefits listed above substantiated [46] who anticipated that bacteria would be found useful both as food and as biological control agents of fish diseases and activators of the rate of nutrient regeneration in aquaculture. Zong-fu et al. [12] stated that potential probiotic microorganisms must be able to colonize the fish intestinal mucosa and produce materials, which are eco-friendly to the host but antagonistic to pathogens. Furthermore, optimal diet utilization by the host animal has been ensured with the use of probiotics, which stimulate the multiplication of gut micro flora in the host fish. It should be noted that an application of probiotics into the water and ponds may also have a positive effect on fish health by improving the water quality, since they modify the bacteria composition of the water and sediments.

5.1. Application of probiotics as biological control agents in aquaculture

Probiotics have been applied in various aspects of aquaculture with promising results, especially in shrimp production [47]. *Luminous vibrio* has also been reported to be completely eliminated from the water column and from the sediment of ponds when probiotic strains selected for their inhibitory effect were used [48]. Hence, disease problems could be overcome by applying probiotic biotechnology, which is an application of microbial ecology [47]. Probiotics are expected to have a direct involvement in nutrients or vitamins [26]. They also enhance the growth of fish [49]. Lack of data on the efficacy of probiotics in commercial aquaculture is still affecting the sustained use of probiotics [50]. Most studies on the effects of probiotics on cultured aquaculture animals have emphasized a reduction in mortality or improved resistance against putative pathogens [51]. Probiotic can be added to the host or its ambient environment in several ways such as:

- Addition to artificial diet
- Addition to culture water
- Bathing
- Addition via live food

Probiotics could be provided to animals in different ways depending on the aim and objective of the study. However, the best method of administration is continuous feeding. This would ensure that the probiotics is present in the gut in a large number and able to metabolize and produce its probiotics effects.

5.2. Probiotic use in fish eggs, larvae, juvenile, and adult fish

The need to control the micro biota in hatching incubators through the alternative means in reducing the use of antibiotics needs to be adequately emphasized. Fish larvae may ingest substantial amount of bacteria by grazing on suspended particles and egg debris [52]. Ringo

and Gatesoupe [26] added lactic acid bacteria (LAB) to larvae of some fish species and a significant reduction of larval mortality was recorded when the larvae were challenged with pathogenic microorganisms (*Vibrio*). Ref. [32] fed lactic acid bacteria to *Atlantic Cod* fry to look at the effect of lactic acid bacteria on the growth and survival rate of Atlantic Cod fry. The experimental fish were given short term bathing in a bacteria suspension of probiotic [27]. Long term exposure in the rearing water led to the reduction in mortality of fish [9]. Ref. [23] selected several strains with a positive effect on the survival and growth of artemia juvenile.

5.3. Improving the immune response of the fish larvae

The level of immune response exhibits by the host animals greatly depend on the immune stimulants such animal is able to produce. Immune stimulants are produced to resist or combat any foreign body or objects intended to infect such animal. The immune systems of fish larvae are less developed; therefore, depend on nonspecific immune response to fight against infection [21]. Observations obtained in experiments with warm-blooded animals indicate that probiotic (lactic acid bacteria) administered orally increased resistance to enteric infections [49]. There are many reports that bacterial compounds act as immune stimulant in fish; however, it is not clear whether bacteria administered as probiotic could have a beneficial effect on the immune response of cultured aquatic species [8]. The role of lactic acid bacteria (LAB) within the digestive tract of endothermic animals and humans has been extensively studied [30]. Few authors have tested in-vivo, the protection conferred by probiotics in fish experimentally infected with pathogens. Bernet et al. [53] found that *Lactobacillus* strains isolated from rotifers increased the resistance of Turbot larvae against a pathogenic *Vibrio* species. Gildberg at al. [32] demonstrated that *Carnobacterium* divergens decreased the mortality rate of Atlantic Cod fry challenged with *Vibrio anguillarum.* Douillet and Langdon [54] also reported that *Carnobacterium* administered to fry and fingerlings of Atlantic salmon reduced the mortality caused by *Aeromonas salmonicida, Vibrio ordalli, and Yersinia ruckeri.* The role of lactic acid bacteria as immune-modulators improves non-specific defenses and is well-known for mammals [31]. Villamil [55] stated that this role has to be determined for fish. Most studies with probiotics conducted to date with fish have been undertaken with strains isolated and selected from aquatic environment and cultured animals.

5.4. Improvement of water quality

Water quality has been recorded to be improved at the addition of probiotics especially, *Bacillus species*. The rationale behind this is that gram positive *bacillus species* are generally efficient in converting organic matters back to CO_2 than gram negative bacteria [8]. Probiotics has also found its usage in water purification, especially with the culture of nitrifying bacteria in bio filters. Nitrifiers are responsible for the oxidation of ammonia to nitrite and subsequently to nitrate. The nitrifying cultures could be added to the ponds or tanks when an incidental increase of ammonia or nitrite levels is observed. Besides ammonia, nitrite toxicity is a common problem in fish culture especially in stagnant pond and re-circulatory system [56].

5.5. Improvement in fish growth

Inclusion of probiotics in the diets of fish species such as hybrid striped bass (*Morone chrysops xm saxatilis*), *Oreochromis niloticus*, catfish, and carp could improve the growth performance, body length, weight gain, and feed conversion ratio (FCR) of fish species [57]. Probiotics could also improve the body composition of fish fed with it. The addition of probiotics in the fish diets was reported to reduce the mortality rate. Gatesoupe [30] showed that turbot (*Scophthalmus maximus*) larvae fed with rotifers enriched with lactic acid bacteria had improved resistance against pathogenic vibrio infection, while noninfected fish showed slight increase in mortality when the level of lactic acid bacteria in the feed was too high.

5.6. Improve the hematology of fish

The hematological parameters of fish have been reported to be improved with the addition of probiotic bacterial into the diets of the experimental fish. For instance, the red blood cell counts (RBC) and white blood cell (WBC) of experimental fish were reported improved after being fed with probiotic bacterial [58]. Probiotics actively stimulate the proliferation of lymphocytes (both B and T cells) and further immunoglobulin production in fish [59]. Application of hematological techniques is, therefore, valuable in fish biology for the assessment of fish health and stress response. In the hemoglobin, oxygen is bound and released easily by iron (Fe) action contained in the hemoglobin molecule as blood transverse the pulmonary capillaries. Red blood cells (RBC), mean corpuscular hemoglobin (MCH), and hematocrit (HCT) have been reported by Adeyemo et al. [60] to indicate secondary responses of an organism to irritants. O'Neal and Weirich [61] describe decrease in erythrocytes to be the major and reliable indicators of various sources of stress in fish. Decrease in white blood cells (WBC) indicates vulnerability to stress and infection [58]. Decrease in red blood cells (RBC) indicates reduction in level of oxygen (O_2), which is being carried to the tissue and carbon dioxide (CO_2) that is returned to the lungs. It also indicates malnutrition in animal. Decrease in mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (McHc) indicates anemia [58]. Differential counts of neutrophils and monocytes give the level of protection against bacterial invasion, while lymphocytes determine the level of immunity. High platelet values show that the fish is likely to withstand and get healed quickly from bruises or wounds, which could be acquired from fight or overstocking. Heterophil/Lymphocyte ratio is a reliable indicator of stress associated with injury [62]. Increase in heterophil/lymphocyte (H/L) ratio indicates stress. Probiotics also actively stimulate the proliferation of lymphocytes (both B and T cells) and further immunoglobulin production in fish [59].

Serum biochemistry deals with the level of various enzymes, minerals, and proteins in the blood. Biochemical values are sometimes variably or invariably affected by blood, sex, age, environment, nutritional status, and experimental factors. Serum is the preferred sample for chemistry analysis, although plasma is often used because of the difficulties of obtaining two samples from one animal. The yield of plasma from a sample is usually greater than that of serum. If plasma is used for the analysis, then the auto coagulant should be considered in result interpretation. Choudhury et al. [63] discovered that dietary supplements of ribonucleic acid significantly influenced the total serum, protein, albumin, and globulin of the experimental fish

(*Labeo rohita*). The highest plasma protein concentration was recorded in fish fed 25% yeast-based diet [64]. Kobeisy et al. [65] studied the roles of 0, 5, 10, and 20% dietary live yeast on the serum glucose of *Oreochromis niloticus* for 13 weeks. They recorded a significant increase in the serum glucose concentration, compared to the control group.

5.7. Stress reduction in fish

Stress is referred to as "the non-specific response of the body to any demand made upon it." Stresses are additives and increase the susceptibility of animals to disease while decreasing their growth rate and feed conversion efficiency [66]. The degree to which stress affects any particular fish is determined largely by the severity of the stress, its duration, and the health of the fish. Reduced or negative growth is commonly observed during stressful periods, while growth rates or derived parameters are often considered reliable indicators of stress and welfare [67]. Fish under intensive culture conditions are exposed to a variety of stressors owing to the economic realities of large scale production [68]. To enhance production, farmers often increase rearing densities beyond system capacities. Rearing at high density can cause stress through deterioration in water quality, overcrowding, or adverse social interactions [69]. High rearing density adversely increases fish susceptibility to disease, possibly as a result of chronically elevated cortisol levels, which have immune-suppressive and catabolic actions in fish [70]. The common symptoms of stress include: gasping at the surface for oxygen, lack of appetite for food, abnormal swimming position, and fish disease. Stocking density is one of the key factors determining profitability and economic sustainability of a fish farm. Meanwhile, farmers often increase rearing densities to intensify production [71] and these suboptimal conditions may result in chronic stress in fish culture [72]. Three types of stress indicators can be detected in fishes: release of corticosteroid hormones (for example cortisol) into blood circulation [73], changes in hematological parameters, and the whole animal performance like growth and survival rate [68].

Hormonal and blood parameters have frequently been used as indicators of stress in sturgeons [74]. Stocking density has been studied in many bony fishes [72]. Stocking density is one of the most important factors in aquaculture because it directly influences survival, growth, behavior, health, feeding, and production of fish under farmed conditions [75]. The effect of stocking density as a major factor affecting fish growth has been the subject of many studies [76]. Hematological parameters are important indices related to response of fish to different environmental conditions. They are considered as important stress indicators in estimating reactions of fish to various environmental conditions and assessment of its general physiological status [77]. The level of hematological and growth indices in fishes is an important parameter to evaluate the stress responses to various environmental conditions [78]. Many studies have confirmed the significance of the hematological parameters to assess the response of organism to the environment condition and their importance for estimating its general health condition and possible effect of exposure to stressors [79]. Stocking density is considered an environmental stressor in aquaculture [80]. This constitutes an important item in any fish culture operation. The result of the improvement in output with respect to stocking density is essential in an intensive production system [81] with the objective of profit maximization.

Production economics revealed that high stocking density of 40 fingerlings/m^3 gave the highest profit index and best cost ratio. At high stocking density (40 fingerlings/m^3), raising of *C. gariepinus* is more profitable [82]. Therefore, to increase fish production, appropriate environmental conditions must be provided [83]. Sohrab et al. [84] reported that growth and nutritional indices were appropriate indicators in assessing the impact of the induced stresses in the Caspian roach larvae owing to stocking density. Many studies have mentioned the importance of the hematological indices to evaluate the stress status of fish as a result of stocking density [84]. Hematological parameters such as hematocrit (HCT), hemoglobin (HB), number of red blood cells (RBC), eosinophil (EOS), and heterophil (HET) in Beluga (*Huso huso*) were not affected by increasing stocking density [85]. Binukumari and Anbarasi [86] recorded decreased trend in neutrophil numbers, RBC, and WBC (white blood cell) count in the stressed fish.

5.8. Accelerates wound healing in fish

Wound occurs when the integrity of any tissue is compromised for instance: skin breaks, muscle tears, burns, or bone fractures. This is very common with scale less fish such as catfish. Fish skin is divided into three layers namely: epidermis, dermis, and hypodermis. The skin is the outer covering of an animal, which forms a barrier against harmful microorganisms and chemicals entering the body. It has the ability to constantly renew itself after injury. It is highly vulnerable to injury owing to its position outside the body of the animal. The process of wound healing depends on how deep the wound is. Healing of wounds is characterized by synthesis of collagen. Wound-healing studies have been carried out on *Heterobranchus bidorsalis* juveniles [15], rainbow trout [87], channel catfish [88], and Nile tilapia [89]. Erazo-Pagador and Din [20] reported *Clarias gariepinus* fed with diets with dietary ascorbic acid had more rapid and complete wound healing. Histological examination by Erazo-Pagador and Din [20] revealed that at 14 days after wounding, fish fed with diets without ascorbic acid had normal epidermis and dermis but muscle tissues were still regenerating, whereas fish fed with diets containing ascorbic acid had normal epidermis, dermis, and muscle tissues. Rapid wound healing is especially important in the intensive culture of African catfish. This is because these species behave aggressively, have no scales, and have strong pectoral spines that can inflict wounds, especially at high stocking densities [20].

6. Conclusion

Having mentioned all the benefits that could be derived from using probiotics in fish production, it will be very imperative to embrace the use of this eco-friendly method in fish culture.

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Probiotic has been used for centuries especially in fermented dairy products since Metchnikoff associated the intake of fermented milk with prolonged life. Probiotics confer many health benefits to humans, animals, and plants when administered in proper amounts. These benefits include the prevention of gastrointestinal infections and antibiotic-associated diarrhea, the reduction of serum cholesterol and allergenic and atopic complaints, and the protection of the immune system. Furthermore, the proper usage of probiotics could suppress *Helicobacter pylori* infection and Crohn's disease, improve inflammatory bowel disease, and prevent cancer.

In this book, we present specialists with experience in the field of probiotics exploring their current knowledge and their future prospects.

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