UNIVERSITÀ DEGLI STUDI DELL'AQUILA

DIPARTIMENTO DI MEDICINA CLINICA, SANITÀ PUBBLICA,

SCIENZE DELLA VITA E DELL'AMBIENTE

DOTTORATO DI RICERCA IN SCIENZE DELLA SALUTE E DELL'AMBIENTE

XXXII ciclo

Curriculum

Medicina traslazionale

Titolo della tesi

EFFECTS OF PROBIOTIC SELECTED STRAINS ON IN VITRO EPITHELIAL CELL MODELS

SSD MED/04 – Patologia generale

Dottoranda

Dr.ssa Armela Kapaj

Coordinatore del corso

Chiar.ma Prof.ssa M. Grazia Cifone

Tutor

Chiar.ma Prof.ssa M. Grazia Cifone

A.A. 2018/2019

Abstract

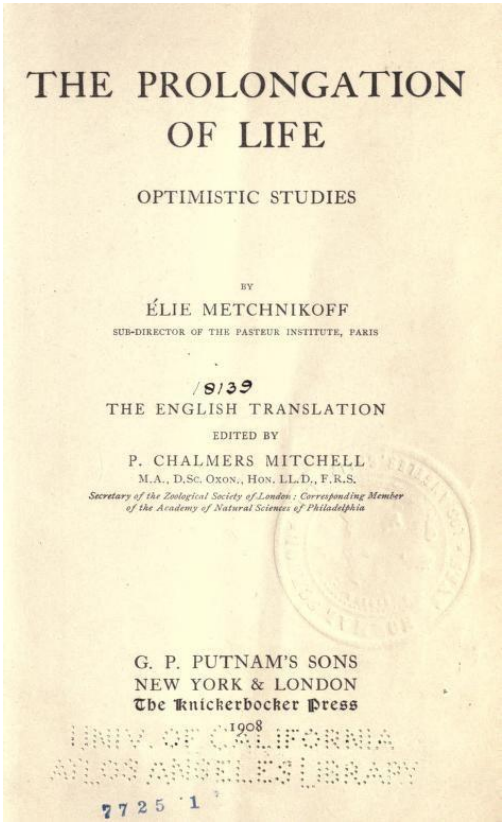
La somministrazione orale di probiotici ha dimostrato di esercitare effetti positivi nei disordini sia intestinali che extra-intestinali. Inoltre, un numero crescente di lavori scientifici evidenzia come i probiotici possano apportare benefici a livello cutaneo non solo dopo assunzione orale ma anche in seguito ad applicazione topica. Lo scopo del progetto di dottorato è stato quello di valutare gli effetti in vitro di ceppi selezionati di probiotici su due differenti modelli di epitelio per valutare la loro capacità di influenzare la riparazione tissutale. A tale scopo, è stato inizialmente analizzato l'effetto della frazione solubile da lisati di ceppi selezionati di probiotici su un modello di guarigione di ferita in vitro. Sono stati identificati i ceppi in grado di influenzare la velocità di riparazione, con particolare attenzione ai due principali processi coinvolti nella guarigione delle ferite: proliferazione cellulare e migrazione. E stato valutato il coinvolgimento del nitrossido nel processo di accelerazione indotto dai diversi ceppi batterici analizzando, in particolare, l'espressione e l'attività dell'enzima nitrossido sintasi II . I campioni di S. thermophilus, L. plantarum e L. acidophilus, erano in grado di favorire la riepitelizzazione dei monostrati feriti di HaCat. Al contrario B. longum, B. infantis e B. breve inibivano il processo di riparazione del monostrato, mentre L. bulgaricus non aveva alcun effetto.
Le proprietà in grado di favorire o inibire la riparazione del monostrato erano associate alla capacità dei probiotici utilizzati di up- o down -modulare l'espressione e l'attività della NOS2.
Questi risultati indicano come la scelta del probiotico giusto sia di importanza cruciale, poiché gli effetti di questi batteri sono altamente ceppo-specifici.
Il secondo modello utilizzato è un modello di barriera epiteliale basato sulle cellule del carcinoma del colon umano, le CaCo-2. Le CaCo-2 sono ampiamente utilizzate per la loro capacità di differenziarsi spontaneamente in un monostrato polarizzato quando coltivate su inserti porosi, assumendo molte caratteristiche morfo-funzionali dell'epitelio intestinale, ed esprimendo proteine delle Tight Junctions simili a quelle dell'intestino tenue. Gli effetti di una formulazione probiotica (VIVOMIXX), sono stati analizzati sulla resistenza elettrica trans-epiteliale (TEER), metodo utilizzato per vautare l'integrità di barriera in vitro, sul flusso di destrano fluoresceinato (FD4), metodo utilizzato per valutare la permeabilità paracellulare di un monostrato polarizzato e sull'espressione delle proteine delle tight junctions, come Zonulina-1 (ZO-1) e occludina, in presenza o assenza di un danno del monostrato cellulare causato da stress termico. Il Vivomixx è in grado di contrastare la perdita dell'integrità della barriera e l'aumento della permeabilità paracellulare indotti dal trattamento con calore. Inoltre, il Vivomixx induceva un aumento dei livelli di occludina, anche in presenza di stress da calore, dimostrando di avere un effetto protettivo contro le lesioni intestinali. I nostri dati supportano anche come questo modello in vitro possa essere utile per testare formulazioni probiotiche allo scopo di rilevare proprietà funzionali in vitro che potrebbero essere clinicamente rilevanti.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 PROBIOTICS

Probiotics are defined as live microorganisms with health-promoting properties. The term "probiotics" was used for the first time by Lilly and Stillwell in 1965, but their positive effects were previously illustrated more than a century ago by Elie Metchnikoff, the Russian immunologist and Nobel Prize for Physiology or Medicine 1908. Metchnikoff associated the constant consumption of yogurt to the delay of the aging process and the promotion of a long life. In his book "The Prolongation of Life" (1907) (**Figure 1**), he indicated that putrefactive bacteria present in the gut produce toxins and other noxious molecules to the host.



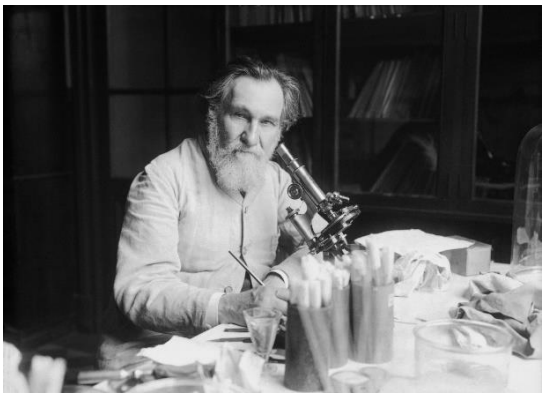


Figure 1. "THE PROLONGATION OF LIFE, OPTIMISTIC STUDIES", Elie Metchnikoff, 1908

Currently, FAO and WHO (Food and Agriculture Organization of the United Nations and the World Health Organization) state probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1].

At birth, diverse commensal microorganisms begin colonizing the human body and remain during the course of life [2] (**Figure 2**).

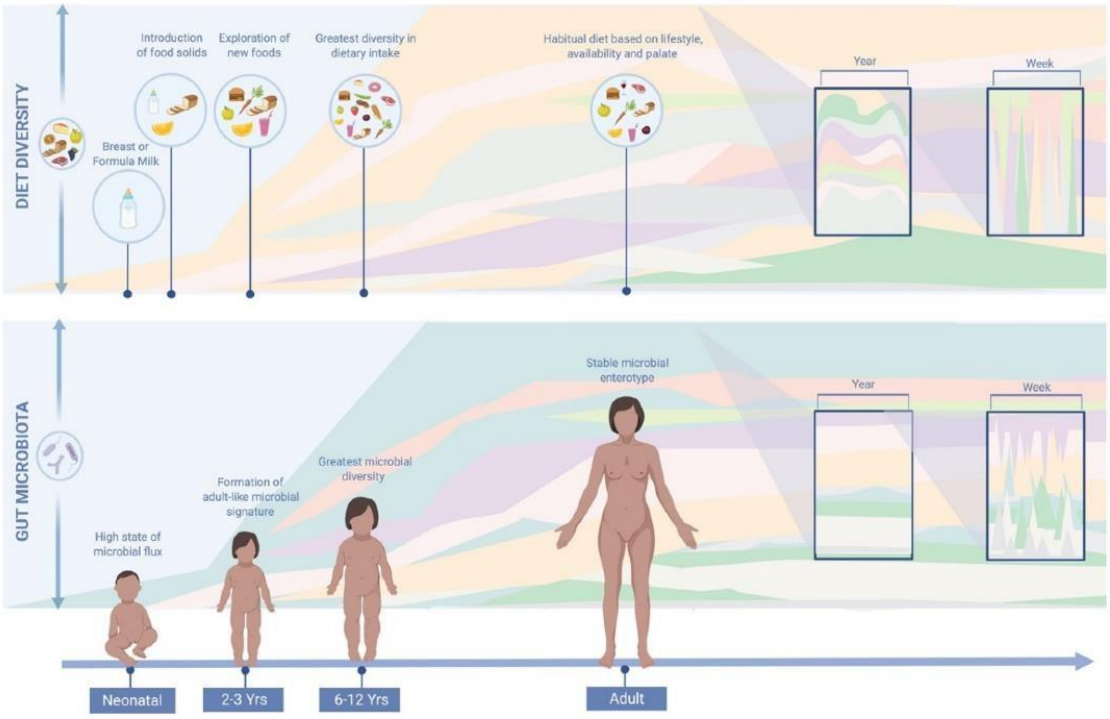


Figure 2. Comparison of diet and gut microbiota variations throughout life. Habitual diet plays a role in shaping the gut microbial environment, and hence, microbial composition. Dietary diversity has been associated with microbial diversity. Throughout the year, the human diet tends to display a cyclical seasonal pattern due to seasonal availability and dietary preferences. Large day to day variations in diet are not reflected in the gut microbiota, suggesting that overall dietary habits have a greater impact on gut microbial composition [2].

Commensal bacteria present in symbiotic communities are able to survive without harming host health whereas hindering pathogenic microorganisms which can alter host barriers and contribute to disease pathogenesis [1]. Of interest, a noteworthy co-evolution and functional integration have been established between microbiota and the human body indicating that the microbiome plays a main role on multiple physiological functions such as defense against infections and modulation of the immune response. The importance of in maintaining host health was recognized early in gut microbiota research. It was demonstrated that germ-free animals are more sensitive to pathogen colonization [3], have impaired mucosal wound healing [4], and are more prone to chemical intoxication [5].

There is growing evidence that human microbiota can exert effects at sites quite distant from the niches in which they reside. For instance, it was shown that gut bacteria can affect memory and anxiety of animal models [6]. Interestingly, low microbiota

diversity in the gut has been associated with increased asthma at school age [7]. In this regard, probiotic action is not limited to the gut, but also imparts their beneficial effects at distant sites and organs. Thushara et al. [8] reviewed experimental and clinical studies of probiotic action against cardiovascular disorders, and an update of their effects on metabolic diseases was provided by Aggarwal et al. [9]. Moreover, it has been demonstrated that probiotic consumption results in production of anti-inflammatory agents that promote bone health and integrity and protect against primary (estrogen-deficiency) and secondary osteoporosis [10]. Similarly, probiotics have also been tested for their wound-healing abilities, protection against UV-induced photodamage, and also for alleviation of symptoms in skin diseases, such as dermatitis and psoriasis [11]. One of the most exciting research areas is their potential functional impact on the nervous system and neurological diseases. Studies in animal models have shown that consumption of probiotics can lead to decreased rate of stress- and anxiety-related symptoms [12]. Furthermore, probiotics have been documented in preclinical studies to suppress pro-inflammatory and oxidative damage responses in the brain, and thus, minimize the symptoms of neurodegenerative and demyelinating diseases [13]. However, as clinical evidence that supports probiotic action at distant sites is limited, no specific recommendations and medical guidelines for appropriate administration are available [14]. Due to important local and systemic effects of gut microbiota, approaches that manipulate its composition to improve host metabolic, immunological, and physiological functions, have become of growing importance. This led to the identification of beneficial bacterial species that belong to symbiotic microbiota and improve host welfare.

The most common microorganisms used as probiotics are lactic acid bacteria (LAB), particularly the genus: *Lactobacilli*, *Streptococci*, *Pediococcus*, *Enterococcus*, *Bifidobacteria*, and some yeast like *Saccharomyces boulardii* [15]. However, not all the bacteria can be probiotic, as they need to be strain-specific. Probiotics have been reported to be beneficial in the treatment or prevention of cutaneous inflammatory conditions [16], prevention and management of diabetes [17], respiratory tract infections [18], various gastro-intestinal (GI) disorders [19,20], urogenital infections [21] and ulcerative colitis [22]. Recent studies also focused on the application of non-

viable probiotic metabolites, termed postbiotics, as a safer alternative to probiotics [23,24,25] (**Figure 3**).

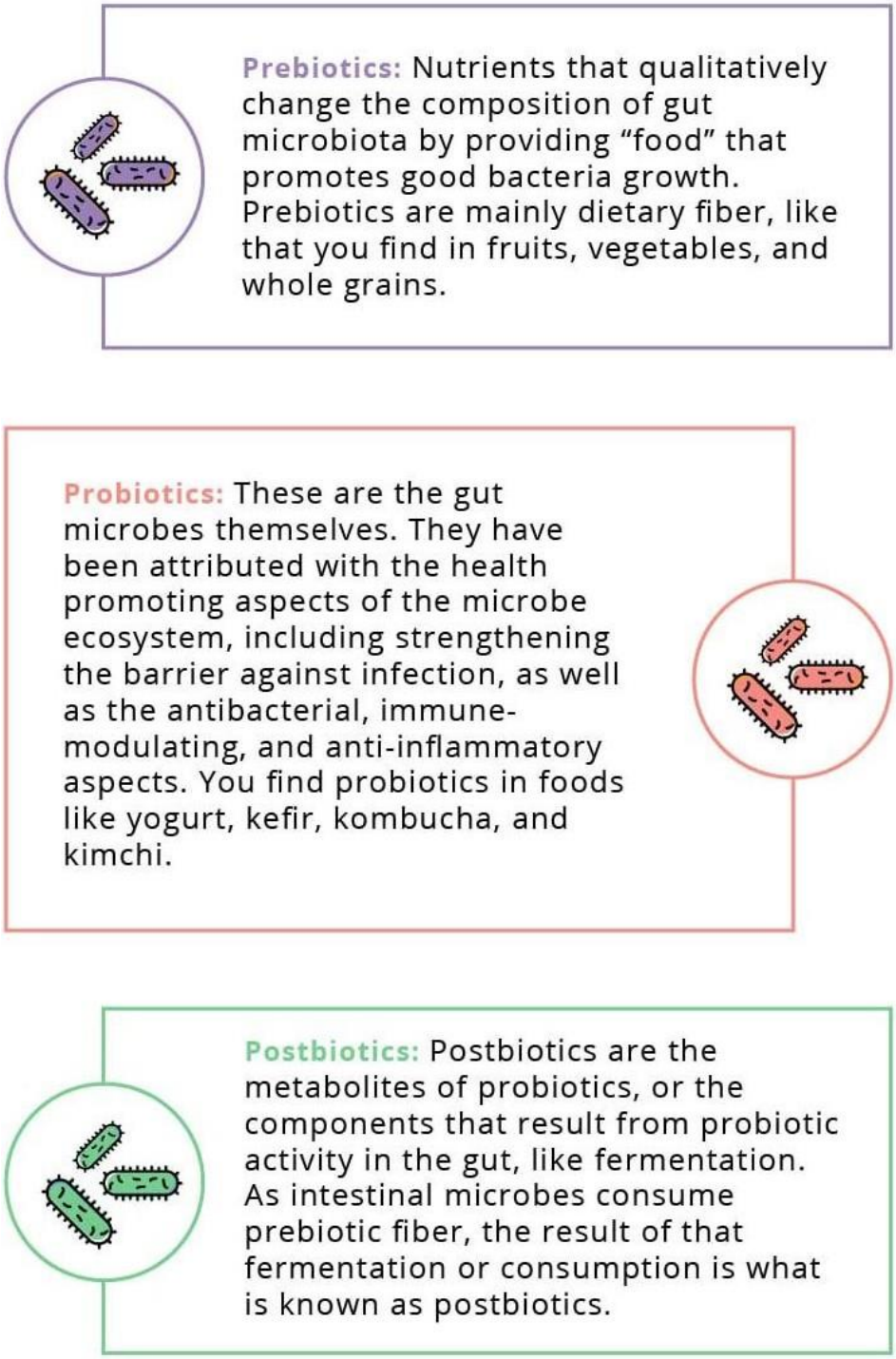


Figure 3. Probiotic versus Prebiotic versus Postbiotic: The definition.

Postbiotics gained special importance in the treatment of inflammatory disorders, where the application of live bacteria bears the risks associated with excessive activation of the immune system. Several articles reviewed their mechanisms of action, which include modulation of the immune system, induction of anti-inflammatory and anti-oxidant responses, competitive pathogen exclusion, as well as production of anti-microbial substances [26,27] (**Figure 4**).

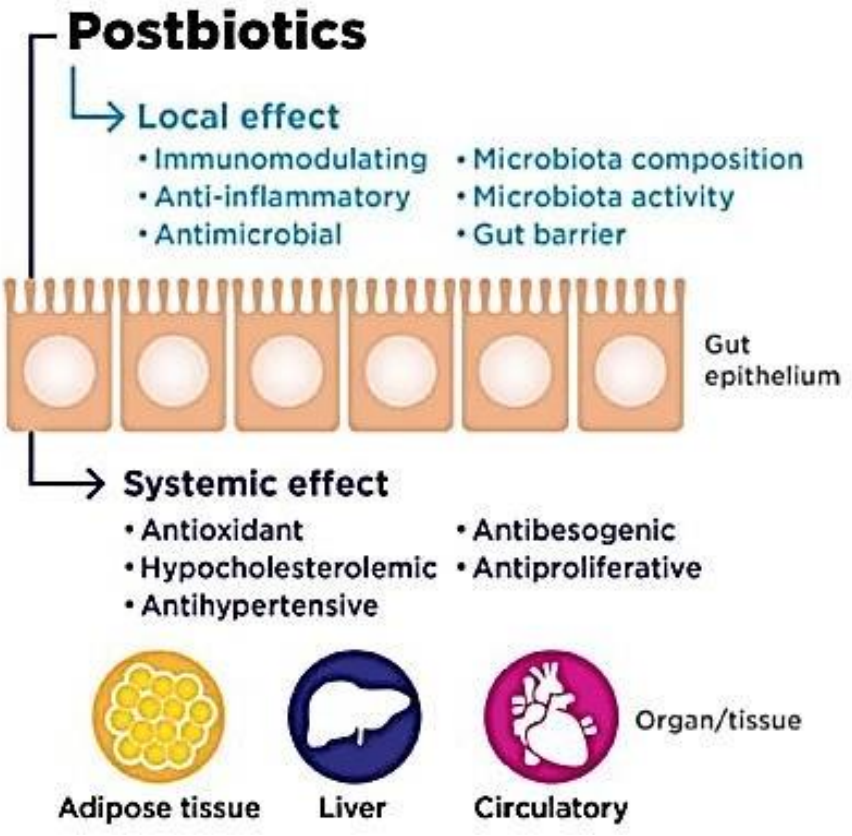


Figure 4. Specific postbiotic have local and systemic effects

1.1.1 Selection criteria and requirements for probiotic strains

According to the suggestions of the WHO, FAO, and EFSA (the European Food Safety Authority), in their selection process, probiotic strains must meet both safety and

functionality criteria, as well as those related to their technological usefulness (**Table 1**). Probiotic characteristics are not associated with the genus or species of a microorganism, but with few and specially selected strains of a particular species [28]. The safety of a strain is defined by its origin, the absence of association with pathogenic cultures, and the antibiotic resistance profile. Functional aspects define their survival in the gastrointestinal tract and their immunomodulatory effect. Probiotic strains have to meet the requirements associated with the technology of their production, which means they have to be able to survive and maintain their properties throughout the storage and distribution processes [29]. Probiotics should also have documented pro-health effects consistent with the characteristics of the strain present in a marketed product. Review papers and scientific studies on one strain may not be used for the promotion of other strains as probiotics. It has to be considered, as well, that the studies documenting probiotic properties of a particular strain at a tested dose do not constitute evidence of similar properties of a different dose of the same strain. Also, the type of carrier/matrix is important, as it may reduce the viability of a particular strain, thus changing the properties of a product [30].

Table 1. Selection criteria of probiotic strains

CRITERION	REQUIRED PROPERTIES
Safety	• Human or animal origin. • Isolated from the gastrointestinal tract of healthy individuals. • History of safe use. • Precise diagnostic identification (phenotype and genotype traits). • Absence of data regarding an association with infective disease. • Absence of the ability to cleave bile acid salts. • No adverse effect. • Absence of genes responsible for antibiotic resistance localized in non-stable elements.
Functionality	• Competitiveness with respect to the microbiota inhabiting the intestinal ecosystem. • Ability to survive and maintain the metabolic activity, and to grow in the target site. • Resistance to bile salts and enzymes. • Resistance to low pH in the stomach. • Competitiveness with respect to microbial species inhabiting the intestinal ecosystem (including closely related species). • Antagonistic activity towards pathogens (e.g., *H. pylori*, *Salmonella* sp., *Listeria monocytogenes*, *Clostridium difficile*). • Resistance to bacteriocins and acids produced by the endogenic intestinal microbiota. • Adherence and ability to colonize some particular sites within the host organism, and an appropriate survival rate in the gastrointestinal system.
Technological usability	• Easy production of high biomass amounts and high productivity of cultures. • Viability and stability of the desired properties of probiotic bacteria during the fixing process (freezing, freeze-drying), preparation, and distribution of probiotic products. • High storage survival rate in finished products (in aerobic and micro-aerophilic conditions).

Table 1. Selection criteria of probiotic strains	
CRITERION	**REQUIRED PROPERTIES**
	• Guarantee of desired sensory properties of finished products (in the case of the food industry). • Genetic stability. • Resistance to bacteriophages.

1.1.2 Probiotic microorganisms

Human probiotic microorganisms belong mostly to the following genera: *Lactobacillus*, *Bifidobacterium*, and *Lactococcus*, *Streptococcus*, *Enterococcus*. Moreover, strains of Gram-positive bacteria belonging to the genus *Bacillus* and some yeast strains belonging to the genus *Saccharomyces* are commonly used in probiotic products [31].

Probiotics are subject to regulations contained in the general food law, according to which they should be safe for human and animal health. In the USA, microorganisms used for consumption purposes should have the GRAS (Generally Regarded As Safe) status, regulated by the FDA (Food and Drug Administration). In Europe, EFSA introduced the term of QPS (Qualified Presumption of Safety). The QPS concept involves some additional criteria of the safety assessment of bacterial supplements, including the history of safe usage and absence of the risk of acquired resistance to antibiotics [32,33]. **Table 2** presents probiotic microorganisms contained in pharmaceutical products and as food additives.

Table 2. Probiotic microorganisms used in human nutrition			
Type *Lactobacillus*	**Type *Bifidobacterium***	**Other Lactic Acid Bacteria**	**Other Microorganisms**
L. acidophilus [(a),∗] *L. amylovorus* [(b),∗] *L. casei* [(a),(b),∗] *L. gasseri* [(a),∗] *L. helveticus* [(a),∗] *L. johnsonii* [(b),∗] *L. pentosus* [(b),∗] *L. plantarum* [(b),∗] *L. reuteri* [(a),∗] *L. rhamnosus* [(a),(b),∗]	*B. adolescentis* [(a)] *B. animalis* [(a),∗] *B. bifidum* [(a)] *B. breve* [(b)] *B. infantis* [(a)] *B. longum* [(a),∗]	*Enterococcus faecium* [(a)] *Lactococcus lactis* [(b),∗] *Streptococcus thermophilus* [(a),∗]	*Bacillus clausii* [(a),∗] *Escherichia coli Nissle 1917* [(a)] *Saccharomyces cerevisiae (boulardi)* [(a),∗]

*[(a)]Mostly as pharmaceutical products; [(b)]mostly as food additives; * QPS (Qualified Presumption of Safety) microorganisms.*

1.1.3 Mechanism of action of probiotics

A significant progress has been observed lately in the field of studies on probiotics, mostly in terms of the selection and characteristics of individual probiotic cultures, their possible use, and their effect on health. Probiotics have numerous advantageous functions in human organisms [34] (**Figure 5**).

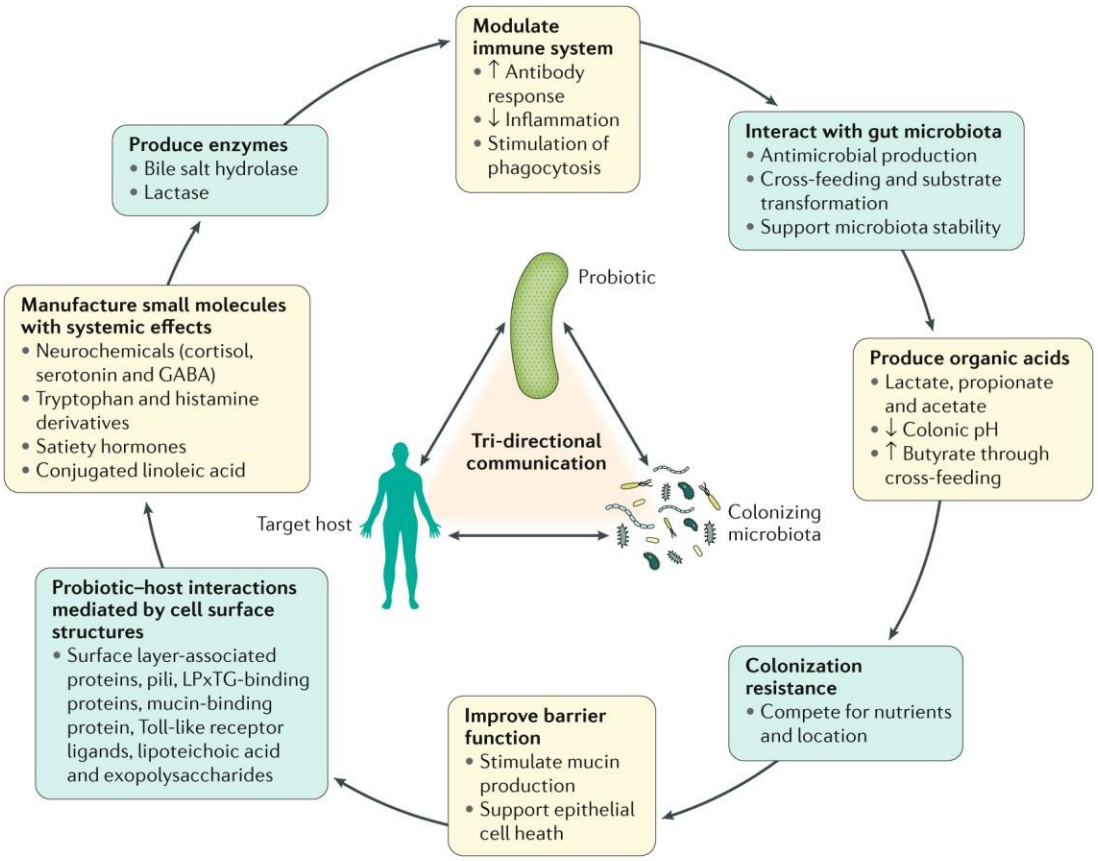


Figure 5. Diverse mechanisms are likely to drive probiotic benefits to host health [34]. In some cases, such as production of antimicrobial products and cross-feeding other resident microorganisms, these mechanisms are driven directly by interactions with the resident microbiota. In other cases, such as direct interaction with immune cells, their effects might be direct via interaction with host cells. Overall, clinical benefits delivered by probiotics could result from the combined action of several mechanisms. GABA, gamma-aminobutyric acid.

Their main advantage is the effect on the development of the microbiota inhabiting the organism in the way ensuring proper balance between pathogens and the bacteria that are necessary for a normal function of the organism [35]. Live microorganisms meeting the applicable criteria are used in the production of functional food and in the preservation of food products. Their positive effect is used for the restoration of natural microbiota after antibiotic therapy [36,37]. Another function is counteracting the activity of pathogenic intestinal microbiota, introduced from contaminated food and environment. Therefore, probiotics may effectively inhibit the development of pathogenic bacteria, such as *Clostridium perfringens* [38],

Campylobacter jejuni [39], *Salmonella enteritidis* [40], *Escherichia coli* [41], various species of *Shigella* [42], *Staphylococcus* [43], and *Yersinia* [44], thus preventing food poisoning. A positive effect of probiotics on digestion processes, treatment of food allergies [45], candidoses [46], dental caries [47], and periodontitis [48] has been confirmed. Probiotic microorganisms such as *Lactobacillus plantarum* [49], *Lactobacillus reuteri* [50], *Bifidobacterium adolescentis*, and *Bifidobacterium pseudocatenulatum* [51] are natural producers of B group vitamins (B1, B2, B3, B6, B8, B9, B12). They also increase the efficiency of the immunological system, enhance the absorption of vitamins and mineral compounds, and stimulate the generation of organic acids and amino acids. Probiotic microorganisms may also be able to produce enzymes, such as esterase, lipase, and co-enzymes A, Q, NAD, and NADP. Some products of probiotics' metabolism may also show antibiotic (acidophiline, bacitracin, lactacin), anti-cancerogenic, and immunosuppressive properties [52,53].

Molecular and genetic studies allowed the determination of the basics of the beneficial effect of probiotics, involving four mechanisms [54]:

1. *Antagonism through the production of antimicrobial substances;*
2. *Competition with pathogens for adhesion to the epithelium and for nutrients;*
3. *Immunomodulation of the host;*
4. *Inhibition of bacterial toxin production.*

The first two mechanisms are directly associated with their effect on other microorganisms. Those mechanisms are important in prophylaxis and treatment of infections, and in the maintenance of balance of the host's intestinal microbiota. The ability of probiotic strains to co-aggregate, as one of their mechanisms of action, may lead to the formation of a protective barrier preventing pathogenic bacteria from the colonization of the epithelium [55]. Probiotic bacteria may be able to adhere to epithelial cells, thus blocking pathogens. That mechanism exerts an important effect on the host's health condition. Moreover, the adhesion of probiotic microorganisms to epithelial cells may trigger a signalling cascade, leading to immunological modulation. Alternatively, the release of some soluble components may cause a direct or indirect (through epithelial cells) activation of immunological cells. This effect plays an important role in the prevention and treatment of contagious diseases, as well as in chronic inflammation of the alimentary tract or of a part thereof [35].

Results of in vitro studies indicate the role of low-molecular-weight substances produced by probiotic microorganisms (e.g., hydroperoxide and short-chain fatty acids) in inhibiting the replication of pathogens [35]. For example, *Lactobacillus* genus bacteria may be able to produce bacteriocins, including low-molecular-weight substances (LMWB—antibacterial peptides), as well as high-molecular-weight ones (class III bacteriocins), and some antibiotics. Probiotic bacteria (e.g., *Lactobacillus* and *Bifidobacterium*) may produce the so-called de-conjugated bile acids (derivatives of bile acids), demonstrating stronger antibacterial effect than the bile salts produced by their host [35,56]. Further studies are necessary to explain the mechanism of acquiring resistance to their own metabolites by *Lactobacillus* genus bacteria. The nutrient essential for nearly all bacteria, except for lactic acid bacteria, is iron. It turns out that *Lactobacillus* bacteria do not need iron in their natural environment, which may be their crucial advantage over other microorganisms [57]. *Lactobacillus delbrueckii* affects the function of other microbes by binding iron hydroxide to its cellular surface, thus making it unavailable to other microbes [58].

The immunomodulatory effect of the intestinal microbiota, including probiotic bacteria, is based on three, seemingly contradictory phenomena [59]:

1. *Induction and maintenance of the state of immunological tolerance to environmental antigens (nutritional and inhalatory);*
2. *Induction and control of immunological reactions against pathogens of bacterial and viral origin;*
3. *Inhibition of auto-aggressive and allergic reactions.*

Probiotic-induced immunological stimulation is also manifested by the increased production of immunoglobulins, enhanced activity of macrophages and lymphocytes, and stimulation of γ-interferon production. Probiotics may influence the congenital and acquired immunological system through metabolites, components of the cellular wall, and DNA, recognized by specialized cells of the host (e.g., those equipped with receptors) [35]. The principal host cells that are important in the context of the immune response are intestinal epithelial cells and intestinal immune cells. Components of the cellular wall of lactic acid bacteria stimulate the activity of macrophages. Those, in turn, are able to destroy microbes rapidly by the increased production of free oxygen radicals and lysosomal enzymes. Probiotic bacteria are also able to stimulate the production of cytokines by immunocompetent cells of the gastrointestinal tract [60,61] (**Figure 6**).

On the other hand, the immunological activity of yeast is associated with the presence of glucans in their cellular wall. Those compounds stimulate the response of the reticuloendothelial system [62]. The last of the abovementioned probiotic effects—inhibition of the production of bacterial toxins—is based on actions leading to toxin inactivation and help with the removal of toxins from the body. Help in detoxification from the body can take place by adsorption (some strains can bind toxins to their cell wall and reduce the intestinal absorption of toxins) but can also result from the metabolism of mycotoxins (e.g., aflatoxin) by microorganisms [63,64,65].

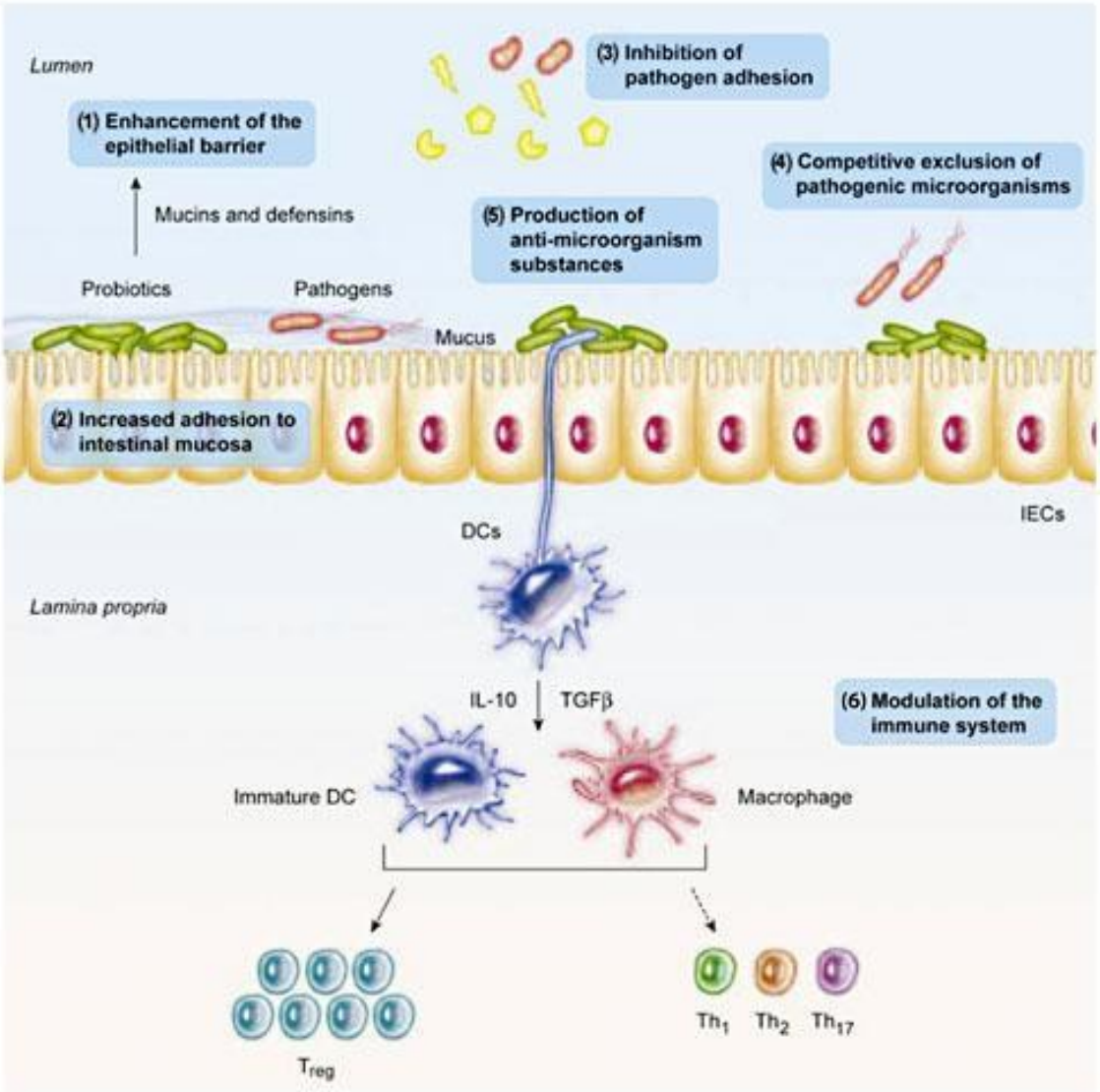


Figure 6: Major mechanisms of action of probiotics [60]

However, not all probiotics exhibit detoxifying properties, as it is a strain-related characteristic. Studies should therefore be conducted to select strains with such characteristics. The effectiveness of some probiotics in combating diarrhea is probably associated with their ability to protect the host from toxins. The reduction of metabolic reactions leading to the production of toxins is also associated with the stimulation of pathways leading to the production of native enzymes, vitamins, and antimicrobial substances [35].

Most probably, all of the abovementioned mechanisms of probiotic action have an effect on the protection against infections, cancer, and the stabilization of balance of the host's intestinal microbiota. However, it seems unlikely that each of the probiotic microorganisms has properties of all four aspects simultaneously and constitutes a universal remedy to multiple diseases. An important role in the action of probiotics is played by species- and strain-specific traits, such as: cellular structure, cell surface, size, metabolic properties, and substances secreted by microorganisms. The use of a combination of probiotics demonstrating various mechanisms of action may provide enhanced protection offered by a bio-therapeutic product [54].

1.2 PROBIOTICS AND GUT

A complex ecosystem of microorganisms is present in the human gastrointestinal tract, acting as commensal can perform several functions useful for the host, such as secrete different nutrients, defend against intestinal pathogens, and affect a physiological immunological response. When this ecosystem appears unbalanced, it's necessary to restore the equilibrium to preserving the health condition of the host by introducing selected bacteria strains as probiotics into diet or as pharmaceutical formulas and functional food. The selected probiotics have several effects due principally to their immune system modulation and anti-inflammatory properties, which justify their use for the treatment of chronic diseases. The clinical studies report numerous benefic applications of probiotics on several diseases as gastrointestinal diseases (e.g., irritable bowel syndrome, gastrointestinal disorders, inflammatory bowel disease, diarrheas) and allergic diseases (e.g., atopic dermatitis) as well as obesity, insulin resistance syndrome, type 2 diabetes, and non-alcoholic fatty liver disease and also in aging. Other positive effects of probiotic use have been reported in different

types of cancer through recommended doses and specific formulations, suggesting that probiotic effects are related to the selected strain, to the combination of multiple strains, dose, and single component.

In the face of widespread diseases and aging societies, the use of comprehension on microbiocenosis of the gastrointestinal tract and the beneficial effect of probiotic bacteria is becoming increasingly important. The eating of pre-processed food (fast food), with excessive amounts of fat and insufficient amounts of vegetables, is another element of the dangerous alteration of human gut microbiota. It's becoming more and more evident that the healthy intestinal microorganisms and its necessary modification with probiotic preparations may protect the individuals against intestinal problems and influence the overall improvement of health [66].

1.2.1 Anti-inflammatory effects of probiotics in intestinal chronic diseases

The chronic intestinal diseases, including inflammatory bowel diseases (IBDs), necrotizing enterocolitis (NEC), and malabsorption syndromes, represent the most serious pathological conditions both in the developed regions and in the developing ones [67,68]. Ulcerative colitis (UC), Crohn's disease (CD), pouchitis, and microscopic colitis, included in inflammatory bowel diseases (IBDs), arise from an alteration of the epithelial barrier function with the addition of the innate immunity and commensal enteric bacteria [66,69,70] (**Figure 7**).

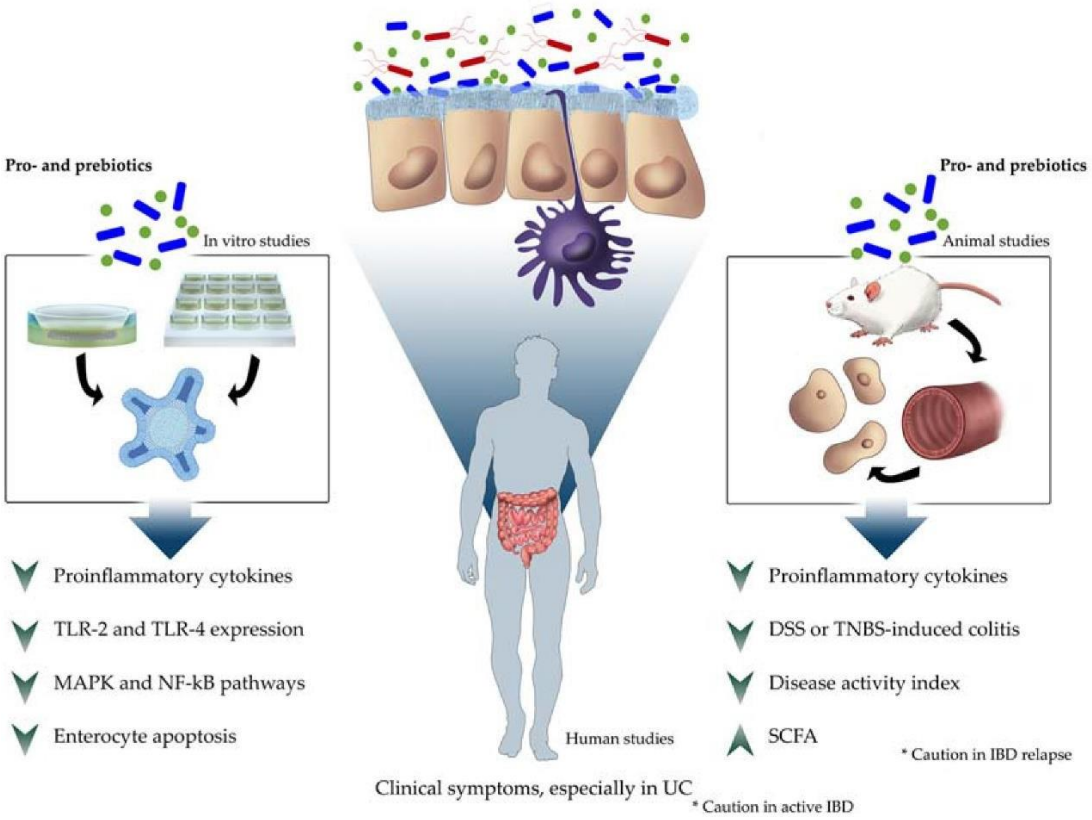


Figure 7. Summary of probiotic anti-inflammatory effects in intestinal chronic diseases in different scientific approaches. DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; SCFA, short-chain fatty acids; TNBS, 2,4,6 trinitrobenzenesulfonic acid; TLR, toll-like receptor; UC, ulcerative colitis [66].

The IBD patients are currently treated with topical or systemic 5-aminosalicylic acids (5-ASA), corticosteroids, or immunomodulators to induce and maintain the remission [71,72]. Clinical benefits are also obtained with selected probiotics thanks to their ability to affect the intestinal microbiota [73,74], and for their anti-inflammatory properties.

In vitro studies

In vitro studies demonstrate strain-specific anti-inflammatory effects of probiotics on several cell models of the intestinal epithelium, in particular using conditions in which the functional epithelial barrier is compromised [75]. Previous reports showed that in UC patients the DCs showed [76,77] an inadequate ability to stimulate the T-cell response, the stimulated T-cells were characterized by high levels of skin-homing markers and augmented secretion of IL-4 was associated with reduced release of IL-22 and interferon (IFN)-γ. When UC-derived DCs were treated with

Lactobacillus casei Shirota (LcS), the normal stimulatory ability was detected through a reduction of the expression of the Toll-like receptor (TLR)-2 and TLR4 [76,77], well-known pattern recognition receptors able to identify several microbial components [78].

Wu et al. [79] reported the effect of *Lactobacillus plantarum* strain CGMCC1258 on IPEC-J2 monolayer model damaged by enterotoxigenic Escherichia coli K88, the epithelial permeability, the inflammatory cytokine expressions, and the levels of tight junction proteins were analyzed. The *L. plantarum* treatment was able to reduce the mRNA levels of IL-8, tumor necrosis factor (TNF-α), and negative regulators of TLRs and the occludin expression at the level of gene and protein [79]. The obtained data suggested that the anti-inflammatory effects of this selected probiotic involved the TLR modulation nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [79]. In another study, the ability of *Lactobacillus delbrueckii subsp. delbrueckii* TUA4408L to affect the activation of the MAPK and NF-κB pathways induced by *E. coli* 987P in PIE cells was investigated. The results showed that *L. delbrueckii* was able to downregulate these mechanisms via the upregulation of TLR negative regulators [80].

Prisciandaro et al. [81] studied the capacity of cell-free supernatants (CFS) from E. coli Nissle 1917 and *Lactobacillus rhamnosus* GG to avoid 5-fluorouracil-induced damage to IEC-6. Indeed, the enterocyte apoptosis and the loss of the intestinal barrier function induced by 5-fluorouracil were hindered (inhibit or prevent) when the selected strains were added before the damage, suggesting a preventive effect on the alteration of intestinal mucosa.

Previously, Bermudez-Brito and his group evaluated in vitro the anti-inflammatory effects of selected probiotic strains using human DCs derived from CD34+ progenitor cells (hematopoietic stem cells) isolated from umbilical cord blood, obtaining a cell system similar to the lamina propria DCs in the gut [82-84]. The intestinal-like human DCs were incubated with Bifidobacterium breve CNCM I-4035 or its CFS, and Salmonella typhi CECT 725, and the TLR-9 gene transcription appeared up-regulated. Also, the pro-inflammatory cytokines and chemokines were reduced when DCs were threatened with S. typhi in the presence of CFS. The similar results were obtained using *Lactobacillus paracasei* CNCM I-4034, and its CFS in the human intestinal DCs damaged with *S. typhi* CECT 725. Moreover, the treatment with *L.*

paracasei CNCM I-4034 induced the TGF-β2 secretion, while the CFS stimulated innate immunity via the TLR signaling activation. Furthermore, when the DCs were challenged with *E. coli* CECT 742, CECT 515, and CECT 729, the treatment with *L. rhamnosus* CNCM I-4036 stimulated the production of TGF-β1 and TGF-β2 and TLR-2 and TLR-4 gene expression also while the CFS augmented the TGF-β1 release and the TLR-1 and TLR-5 gene expression [82-84]. Based on this evidence, it surmises that the inhibitory effect of specific probiotic strains and CFS on the pro-inflammatory cytokine expression is mediated by TLRs.

In vivo studies

Animals

The experimental animal models widely used to evaluate the anti-inflammatory effects of probiotics are represented by spontaneous and chemically induced colitis. In these systems, the animals were supplemented with probiotics, and a downregulated production of inflammatory cytokines, as well as regulatory mechanisms, were observed depending on the strain selected [75].

Dextran Sulfate Sodium. Dextran sulfate sodium (DSS)-induced colitis in Swiss mice represents a reliable model of UC to investigate the anti-inflammatory effects of *Lactobacillus acidophilus, L. plantarum, Bifidobacterium lactis, B. breve*, and inulin. The aforementioned probiotics were able to reduce the colitis severity and the nitric oxide (NO) levels detected in the supernatants of peritoneal macrophage cultures [85].

Atkins et al. [86] observed the effects of *Lactobacillus reuteri* BR11 treatment on experimental animal IBD model obtained by 2% DSS administration. In particular, *L. reuteri* BR11 or *L. reuteri* BR11 mutants deficient in the cystine-uptake system were supplemented for 12 days. When the wild-type strain was administrated, the IBD severity was partially reduced, while the treatment with mutant strain was not able to alleviate DSS-induced colitis.

Cui et al. [87] investigated the therapeutic effects of *Lactobacillus fermentum* CCTCC M206110, *Lactobacillus crispatus* CCTCC M206119, and *L. plantarum* NCIMB8826 on experimental colitis in BALB/c mice treated with DSS. The results showed that *L. fermentum* CCTCC M206110 treatment caused a reduced weight loss,

colon length shortening, disease activity index scores, and histologic scores, while the *L. crispatus* CCTCC M206119 treatment provoked greater weight loss and colon length shortening, histologic scores, and more severe inflammatory infiltration. The administration of *L. plantarum* NCIMB8826 enhanced the weight loss and colon length shortening, but it was not able to affect the disease activity index and histologic damage in the colitis model [87]. Moreover, the *L. crispatus* CCTCC M206119 supplement worsened DSS-induced colitis, while *L. fermentum* CCTCC M206110 effectively improved DSS-induced colitis.

Wong et al. [88] previously studied the effect of the intrarectal administration of mouse cathelin-related antimicrobial peptide (mCRAMP) on DSS-induced colitis. The results demonstrated that the treatment maintained the mucus layer and decreased the pro-inflammatory cytokine secretion. In this study, a mutant of *Lactococcus lactis* NZ3900, able to produce mCRAMP, was used in a murine model of DSS-induced colitis for seven days. The treatment with cathelicidin-transformed *L. lactis* improved the clinical symptoms, maintained crypt integrity and the mucus content, and significantly decreased the number of apoptotic cells, myeloperoxidase (MPO) activity, and malondialdehyde level.

Hong et al. [89] investigated a combination of *Lactobacillus brevis* HY7401, *Lactobacillus* sp. HY7801, and *Bifidobacterium longum* HY8004 in an acute DSS-induced colitis model for a week. The results reported an increase of the acetate, butyrate, and glutamine levels, in addition to reduced levels of trimethylamine, in the feces of the probiotic group when compared to the DSS-alone-treated mice. The data demonstrated that the benefic probiotic effects against DSS-induced colitis were based on the modulation of the gut microbiota.

E. coli Nissle, 1917 was tested in a mouse model of reactivated colitis. A mouse model of reactivated colitis obtained by adding DSS for five days and by subsequent exposure to DSS after two weeks later was used to evaluate the effect of *E. coli* Nissle, 1917 administration. In this animal model following probiotic administration, the intestinal anti-inflammatory effects, attenuated colitis reactivation, as well as a reduction of the expression of pro-inflammatory cytokines and increased intestinal mucin-like and zona occludens-1 expression were observed [90]. Moreover, the effects of *L. rhamnosus* NutRes 1 and *B. breve* NutRes 204 were analyzed on a DSS-induced

chronic murine colitis model induced by two DSS treatment cycles with a 10-day rest period. After the first DSS treatment cycle, probiotic exposure was administrated and continued until the end of the experiment. *L. rhamnosus* NutRes 1, but not *B. breve* NutRes 204, rapidly and effectively improve the DSS-induced bloody diarrhea during the resolution phase. However, *L. rhamnosus* supplementation caused an augmented expression of TLR2, TLR6, chemokine (C-C motif) ligand 2, IL-1β, TNF-α, and IL-6 in DSS-treated mice. These results suggest caution in the use of probiotics in the relapse stages of IBD [91].

The administration of capsules with bifidobacteria, lactobacilli, and *Streptococcus thermophilus* DSM24731 to mice exposed to 5, 10, and 15 cycles of DSS was able to reduce the disease activity index score and colon inflammation, as well as the histological alterations and the incidence of colonic dysplastic lesions in the three periods studied. Moreover, the probiotic mixture induced a reduction of the proliferating cell nuclear antigen labeling index and TNF-α, IL-1β, IL-6 secretion, and cyclooxygenase (COX)-2 expression, and increased IL-10 levels in colon tissue in the three periods assayed [92]. Also, when the rats were exposed with DSS for seven days, the probiotic formulation showed anti-inflammatory properties, such as decreasing the disease activity index, MPO activity, iNOS, COX-2, NF-κB, TNF-α, IL-6, and p-Akt expression, and augmentation IL-10 expression in colonic tissue. Also, the probiotic treatment decreased TNF-α and IL-6 and increased IL-10 levels in the serum [93]. In another work, probiotic exposure studied in acute intestinal ischemia/reperfusion injury in adult 129/SvEv mice, was able to reduce local tissue inflammation and injury with a significant concomitant significant reduction in active IL-1β levels and MPO level in the tissue. Active NF-κB levels were significantly higher in the control group, consistent with the tissue inflammation. Inflammation was attenuated by probiotic administration. Finally, the administration of bifidobacteria, lactobacilli, and *S. thermophilus* did not cause any systemic inflammation or lung injury [94].

Zhang et al. [95] investigated the effect of *Bacillus subtilis* R179 in a mouse DSS model of colitis, reporting an improvement of the gut inflammation and dysbiosis.

2,4,6-Trinitrobenzenesulfonic Acid. The effect of *L. plantarum* 21 on inflammatory molecules in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats has been

investigated. *L. plantarum* 21 exposure for 14 days after the induction of colitis, reduced thiobarbituric acid reactive substances (TBARS) and NO, and increased glutathione concentrations. The IL-1β and TNF-α cytokines, were down-regulated, whereas IL-10 has up-regulated in *L. plantarum* 21-exposed rats. Moreover, probiotic treatment reduced the macroscopic colonic damage and histopathological changes induced by TNBS [96]. The effects of lactobacilli and bifidobacteria treatment on TNF-α and TLR4 expression in a rat colitis model stimulated by TNBS were also evaluated. The treatment with probiotics for two weeks did not affect TLR4 and TNF-α expression, while when the exposure was for four weeks, significant reductions were detected in rats treated with probiotics compared with the TNBS control group [97]. Eeckhaut et al. [98] investigated the effects of *Butyricicoccus pullicaecorum* CCUG 55,265 on a rat colitis model with TNBS and a Caco-2 cell model. The results reported that *B. pullicaecorum* treatment caused a significant protective effect as detected with macroscopic and histological criteria and a reduction of intestinal MPO, TNF-α, and IL-12 levels. Also, *B. pullicaecorum* supernatant prevented the increase of the TNF-α- and IFN-γ-induced IL-8 release in a Caco-2 cell model.

Other intestinal inflammation models. An aberrant immune response towards the intestinal microbiome is the main cause of chronic enteropathies (CE). The in vitro effects of the probiotic Enterococcus faecium NCIMB 10,415 E1707 were previously evaluated in a canine cell model. However, the in vivo efficacy was not detected. Dogs with CE were prospectively used and treated with a hydrolyzed elimination diet, in addition to either a synbiotic product containing *E. faecium* NCIMB 10,415 E1707 or a placebo for six weeks. Both veterinary staff and owners were blinded to the treatment. Of the 45 cases recruited, 12 completed the clinical trial. In the project, the symbiotic product was administrated to seven dogs and placebo products to five animals. The clinical efficacy and histology scores did not show a difference between groups or treatments [99]. Casp-1 and NLRP3 gene expression was decreased in the CE samples if compared with the controls. When *E. faecium* NCIMB 10,415 E1707 were *ex vivo* administrated, the NLRP3 expression reduced in the control samples [99]. Another intestinal malfunction mouse model was obtained with lincomycin hydrochloride and the effects of *L. delbrueckii* subsp. bulgaricus were evaluated. The results showed that

L. delbrueckii treatment induced an increase of secretory immunoglobulin A and reduction of pathological intestinal damage [100]. In a rat model of chronic inflammation caused by N,N-dimethylhydrazine, the secretion of IL-2, IL-6, IL-17, and TNF-α, as well as the levels of NF-κB, COX-2, and iNOS, and the depletion of goblet cells were evaluated using *L. plantarum* LS/07 CCM7766 alone or in combination with inulin. The results showed removal of the inflammatory process in the jejunal mucosa due to inhibition of the release of pro-inflammatory cytokines and stimulation of IL-10 cytokine synthesis, while the TGF-β1 levels did not change significantly [101]. *L. rhamnosus* OLL2838, *Bifidobacterium infantis* ATCC 15,697, and *S. thermophilus* Sfi 39 were assessed on the maturation of bone marrow-derived DCs from mice [102]. *S. thermophilus* Sfi 39 basically induced IL-12 and TNF-α, while *L. rhamnosus* OLL2838 stimulated substantial levels of IL-10 and NO secretion [102]. Moreover, *L. rhamnosus* OLL2838 when it was studied on an in vivo model of gluten-specific enteropathy high levels of intestinal IFN-γ, augmented cell apoptosis in lamina propria and decreased intestinal glutathione S-transferase activity were reported. In addition, the total glutathione and glutathione S-transferase activity increased after probiotic treatment, while caspase-3 activity was decreased. However, the recovery of the normal histology and the high intestinal IFN-γ level probiotic strain failed to recover the normal histology and further increased intestinal IFN-γ [102]. In conclusion, Wu et al. recognized a new role of probiotics in stimulating vitamin D receptor (VDR), thus constraining inflammation, by cell models and VDR knockout mice [103]. The probiotics *L. rhamnosus* GG ATCC 53,103 and *L. plantarum* enhanced VDR protein expression in both mouse and human intestinal epithelial cells. In addition, the ability of probiotics in modulating VDR signaling was evaluated in vivo using a *Salmonella typhimurium* ATCC 14,028-induced colitis model in VDR knockout mice. The treatment with probiotic physiologically and histologically protected the mice from colitis, but no effect was detectable in knockout mice. Probiotic treatment also increased the number of Paneth cells, which produce AMPs for host defense [103]. Animal models seem to be more widely employed than cell models for evaluating of probiotic roles. Even if probiotic treatment can ameliorate clinical symptoms, histological modifications, and mucus secretion in the majority of the analyzed studies, some observations suggest that

attention should be taken when the probiotics are administered in the relapse stages of IBD. Moreover, no effects on chronic enteropathies were observed.

Humans

Ulcerative colitis. UC, a chronic IBD, is identified by acute exacerbations of intestinal complications, followed by remissions. Moreover, UC is with unknown etiology, seem to be provoked by an unnecessary immune response to endogenous bacteria in genetically predisposed individuals. Therefore, an appealing therapy for UC consists of manipulating intestinal microbiota to decrease the inflammatory ability to colonize bacteria. Recent studies have reported the efficacy of probiotic therapy in patients with UC. Tamaki et al. [104] evaluated the usefulness and safety of probiotic treatment with *B. longum* 536 in Japanese patients with active UC using an RCT. The probiotic treatment was able to ameliorate clinical symptoms, such as the UC disease activity index and Rachmilewitz endoscopic index, in patients with mild to moderately active UC, however additional studies are needed to explain the efficacy and safety of *B. longum* 536 for UC [104]. To evaluate the ability of probiotic treatment for one year, including a mixture of the strains *Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A, and *Bacillus mesentericus* TO-A, to prevent the relapse of UC in patients who were already in remission, a single-center, randomized, double-blind, placebo-controlled study was performed. The treatment significantly enhanced the relapse rates at three and nine months, but no differences were observed at 12 months. Moreover, using the cluster analysis of fecal microbiota, which consists of the molecular method that compares the diversity and colony structure of microbial complexes, seven subjects were categorized in cluster I; 32 in cluster II, which represents the "appropriate intestinal microbiota"; and seven in cluster III. As a consequence, probiotic treatment could be useful for UC, especially in cluster I patients [105]. Krag [106] evaluated the clinical efficacy of profermin on patients with relapsing UC, through a randomized controlled trial (RCT). The proferin is a food with fermented oats containing *L. plantarum* 299v and other ingredients (i.e., barley malt and lecithin). The probiotic supplementation was able to reduce significantly Simple Clinical Colitis Activity Index (SCCAI) score in patients with a mild-to-moderate flare-up of UC, showing initially a SCCAI score ≥5

and ≤1. Of note, the treatment with *L. plantarum* 299v, safe, well-tolerated, and palatable, showed the ability to cause a significantly clinical decrease in the SCCAI score when compared with placebo treatment [106].

Crohn's Disease. The CD patients have a systemic disorder characterized by genetic susceptibility as an important etiological factor. Several studies have reported differences in the microbiota of subjects with CD, defined by a decrease of anti-inflammatory bacteria and an enhancement of pro-inflammatory bacteria respect to the microbiota of healthy subjects. Some recent studies have investigated the effect of selected strains on CD. Of note, the effects of *E. coli*, a member of the phylogenetic group B2, have been assessed by Petersen et al., along with its association with both CD and UC [107]. Of note, the probiotic *E. coli* Nissle, 1917 treatment shows the same result of the mesalazine in preventing disease flares in UC patients, and also antibiotics can be useful in the treatment of IBD patients. For this reason, these authors evaluated if the treatment with ciprofloxacin for one week, followed by treatment with *E. coli* Nissle, 1917 for seven weeks, or either of these treatments alone, affect the remission rate among patients with UC. Nevertheless, the use of *E. coli* Nissle 1917 as an add-on treatment to the conventional approach for active UC did not cause benefic effects [107]. The effects of capsules with lactobacilli, bifidobacteria, and *S. thermophilus* DSM24731 in avoiding the relapse of CD after surgery were investigated [108]. The relapse after intestinal resection in CD is a relatively common aspect. In this work, within one month of ileocolonic resection and re-anastomosis, CD patients were grouped and randomly treated with capsules versus a placebo. Even if the differences in the endoscopic recurrence rates at day 90 between patients treated with the probiotics strains were not detected, the mucosal levels of inflammatory cytokines (IL-8 and IL-1β), decreased among patients who were treated with probiotics.

1.2.2 Colon cancer and probiotics in an animal model

The changes in the gut microbiota composition due to inflammatory and carcinogenic stimuli may predispose to tumorigenesis [109] (Figure 8).

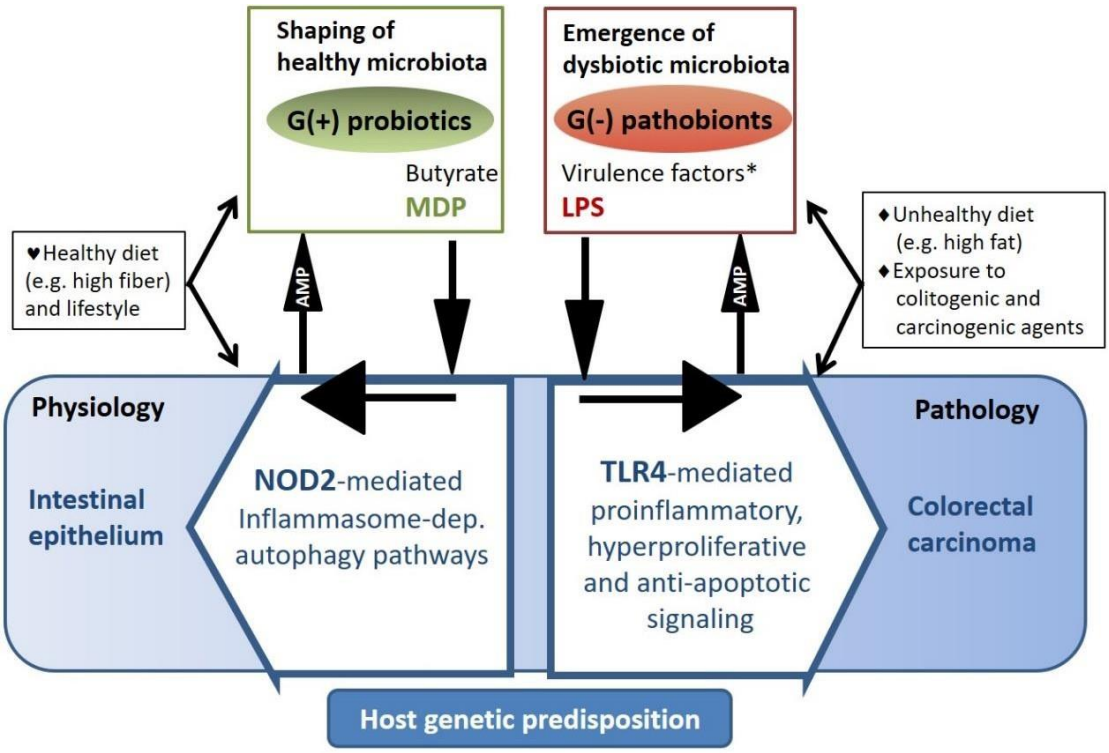


Figure 8. A proposed model of the reciprocal relationship between innate immunity and microbiota in the pathogenesis of colon cancer. During the transition from physiological intestinal epithelium to pathological colorectal carcinoma, two co-existing factors (i.e. innate immune response and microbial composition) synergistically determine the fate of malignant transformation on top of host genetic predisposition. Under a healthy diet and lifestyle, nucleotide-binding oligomerization domain 2 (NOD2) shapes a healthy microbiome via inflammasome-mediated regulation of antimicrobial peptides (AMP). The healthy microbiota contains Gram-positive bacteria as probiotics that produces butyrate and large quantities of muramyl dipeptide (MDP). MDP binding to NOD2 induces inflammasome-dependent autophagy pathways which are involved in the maintenance of epithelial homeostasis. However, with an unhealthy diet and after exposure to colitogenic and carcinogenic agents, overexpression of Toll-like receptor 4 (TLR4) instigates proinflammatory, hyperproliferative and anti-apoptotic signals in colonic epithelial cells after lipopolysaccharide (LPS) binding, and also alters the mucosal defensin level and causes dysbiosis. The dysbiotic microbiota contains Gram-negative pathobionts with virulence factors and outer lipid membranous product LPS. Binding to LPS further increases TLR4 expression on cells, leading to a viscous cycle of activation. The bidirectional aggravation between pathobiont LPS and epithelial TLR4 further contributes to colon tumor development [109**]**.

A study of Zackular et al. [110] detected when the mice were treated with carcinogen azoxymethane (AOM), and after by the inflammatory compound dextran sulfate sodium (DSS), dramatic modifications in the microbial community and significant variations in relative microbial abundances. Moreover, the germ-free mice, when recolonized with the gut microbiota derived from tumor-bearing mice were able to develop more tumors compared with those receiving the microbiota of naïve healthy mice after treatment with AOM/DSS. The obtained data indicated that these modifications directly participate in tumor susceptibility, and the variation of the

intestinal microbiota was an important factor of colon tumorigenesis. Even if this protection system has been well verified, other studies have proposed the role of selected commensal bacteria in restraining inflammation-associated colon tumorigenesis by stimulating several pathways, which are not definitively acknowledged thus far [111,112]. Bacterial metabolites have also been observed to have a defensive effect. Butyrate, for instance, that is a product derived by the fermentative metabolism of dietary fiber, and resistant starches by selected strains of the *Firmicutes* phylum can affect inflammation, epithelial proliferation, and apoptosis [113]. Other studies highlighted the particular role of Lactobacilli in preventing colon cancer in the animal model [114-116], thus supporting the protective effects of *Lactobacillus acidophilus* and *Lactobacillus salivarius* on the precancerous growths and colorectal tumorigenesis in the rat model, respectively. Moreover, another study [117] reported that a specific strain of *Lactobacillus plantarum* was able to inhibit the chemical inducted-colon cancer in the mouse model. It has also been reported that lower intracolonic pH values reduced the proliferation, and then the activity, of putrefactive carcinogenic bacterial enzymes. Of note, Chang et al. [114] demonstrated that the low intracolonic pH displayed by the rats exposed to *L. acidophilus* at a high dosage for ten weeks reduced the intestinal populations of carcinogenic bacteria. In conclusion, all these studies are a clear demonstration that selected strains of probiotics can interfere with exterior conditions, such as the inflammatory process, dysbiosis, or atypical colon metabolism, leading to tumorigenesis.

Microorganisms responsible for or protective of colon cancer

Nowadays, it is complex to recognize a particular bacterial population or variation in their abundance or number of single strains responsible for enhancing tumor susceptibility and development. Several studies have reported that different bacteria, not only *F. nucleatum* or *P. gingivalis*, but also *E. coli*, *B fragilis*, and *E. faecalis*, were augmented in patients with CC, whereas the Clostridiales, Faecalibacterium, Blautia, Bifidobacterium, or Lactobacillus genus were not present [118-120]. Indeed, particular populations or their level cannot be definitively associated with tumorigenesis. Clostridia, for instance, are often recognized as oncomicrobes [112,121], however the complexity of microbiota and the connections between microorganisms (network) in the

gut does not highlight a particular bacterial profile for tumor progression so far. Of course, the gut microbiota can be involved in either health or cancer progression through its inflammatory and proliferative effects likely linked to the context and genetic factors of the host as well. Moreover, there are many available probiotic strains widely used clinically, specific Lactobacilli (but also Bifidobacteria) are well described for their activity and anti-inflammatory role in affecting cytokine production in human dendritic cells [122]. In a study of Kuugbee et al. [123] the *Lactobacillus* administration to mice was able to regulate the expression of Toll-like receptor 2 (TLR2), TLR4, and TLR9, mainly TLR2, and decrease the tumor incidence. Cancer is generally characterized by deficient levels of apoptosis, leading to increased survival of malignant cells. The apoptosis mechanism is very complex, involving many pathways. Gamallat et al. [124] reported that a selected strain of *L. rhamnosus* as a preventive measure could decrease the incidence and diversity of colon tumors by causing cell apoptosis and inhibiting inflammation. A recent study underlined the importance of specific Lactic acid bacteria as the progenitor strain due to its inherent positive properties in generating anti-oxidant enzymes. This study revealed the best anti-cancer effect of mixtures of Lactobacilli by combining different anti-inflammatory mechanisms and IL-10 production in a colorectal cancer mouse model [125]. The study by Verna and Shukla [126] reported that the inhibition of some oncogenic enzymes could be realized by the oral intake of specific probiotics. Some strains of probiotics metabolize and inactivate specific compounds, such as the N-nitroso compounds and heterocyclic aromatic amines [127]. All these activities, in particular, the ability to degrade carcinogenetic enzymes, appear to be relative to the strain type used as probiotics, as well as on the specific host condition. Several species of probiotic bacteria, such as *L. acidophilus*, *L. casei*, *L. plantarum*, *Propionibacterium freudenreichii, Lactobacillus delbrueckii, Bifidobacterium infantis, Bifidobacterium breve, Bifidobacterium longum*, and *Streptococcus thermophilus* are able to produce conjugated linoleic acids from linoleic acid. The fatty acids generated by these bacteria can act into the colonocytes by exerting antiproliferative and proapoptotic functions with locally beneficial effects [128].

Several studies have indicated that the improvement of the quantitative and qualitative profile of the intestinal microbiota could be achieved through regular consumption of probiotics. Thus, the trigger of chronic inflammation and the production

of carcinogenic compounds during intestinal dysbiosis could also be reduced [129-131]. Of note, a study by Liu et al. [131] demonstrated that the regular administration of *L. acidophilus, L. plantarum,* and *B. longum* at a high dosage for about two weeks, was able to increase the diversity and microbial richness in individuals with CRC undergoing a colectomy. Here, the intestinal microbiota composition of patients was similar to that of the healthy subjects. As reported, some intestinal enzymes, such as β-glucosidase, β-glucuronidase, nitrate reductase, azoredutase, and 7-α-dehydroxylase, by converting aromatic hydrocarbons and amines in active carcinogens due to the synthesis of aglycones, phenols, cresols, ammonia, and N-nitroso compounds, can promote cytotoxic and genotoxic effects, thereby contributing to the development of colon cancer. Moreover Hatakka et al. [129] reported that the feeding of selected probiotic bacteria could negatively affect the activity of these enzymes and avoid colon cancer. Several selected probiotics are also able to influence the immune response by activating of phagocytes and contributing to the maintenance of the state of immune-vigilance, which can remove cancer cells in their early stages of development [132,133]. Of note, the immunomodulatory properties depend on strain type; the survival and the persistence in the gut, as well as the posology, can also significantly affect the immune system. Therefore, not all probiotics can influence the immune system and to avoid the occurrence of CC. One promising approach for the colorectal cancer treatment consists of the human microbiota manipulation, and then, the use of specific probiotics. Of note, few initial studies, especially randomized and controlled, to evaluate if manipulation of the microbiota in patients treated for colorectal cancer, may affect outcomes, such as the objective response rate or progression-free survival. However, it has been extensively described that the regular consumption of probiotics can reduce intestinal permeability by varying the distribution of cell junction proteins [134,135] and reducing the amount of potentially carcinogenic substances absorbed and acting negatively on the colonocytes. Patients with CRC treated with a mixture of probiotics (*L. plantarum, L. acidophilus* and *B. longum*) showed improvement of the outcome and increase of the levels of cell junction proteins as well as their distribution along the colonic epithelium [131]. Another factor well investigated in the human tumor is the proapoptotic activity induced by the consumption of probiotics, such as the increase of TNF-α generation [136]. Wan et al. [137] established that the probiotic *L. delbrueckii* was able to induce

the apoptosis of the tumor cells by increasing the expression of caspase-3. In a randomized control trial with colon cancer and polypectomized patients, Rafter et al. [138] showed a decrease of different cancer biomarkers and a reduced genotoxic exposure (IL-2 and INF-gamma) after oral exposure with *L. rhamnosus* and *B. breve* combination. Recently, Kotzampassi et al. [139] reported that the treatment with a probiotic formulation was able significantly to reduce all major post-operative complications in patients undergoing surgery for colon rectal cancer when compared with placebo treatment (28.6% vs. 48.8%, $p = 0.010$, OR 0.42). All these studies cannot demonstrate that probiotic treatment can definitively prevent or heal colon cancer, but they could represent a starting point for further studies in this interesting field. In these studies, there are many potential confounding factors such as BMI, smoking, type of diet, and physical activity. An Italian prospective cohort study described that self-reported yogurt intake had an inverse association with colorectal cancer risk, but the authors evidenced that several confounding factors can affect this result [140]. A very recent and attractive field, the manipulation of the microbiota composition during immunotherapy for CC may lead to the advance of innovative therapeutic approaches in colon cancer. These very preliminary researches are highlighting an important relationship between intestinal microbiota, and the immune system and comprise the possibility of targeting the microbiota for the improvement of anticancer treatment. Certainly, further and deeper studies will be necessary to assess the interaction between the microbiota and colon cancer immunotherapy [141]. Recently, it has become clear that microbiota, particularly the gut microbiota, can affect the response to cancer treatment and the sensitivity to dangerous side effects. The demonstration of the ability of the microbiota to affect chemotherapy, radiotherapy, and immunotherapy with a particular interest in microbial composition, has started to become very remarkable [142,143].

1.3 PROBIOTICS AND SKIN HEALTH

Several human studies have investigated the role of oral administration and topical application of probiotics in a variety of skin conditions [11,144-156] (Figure 9).

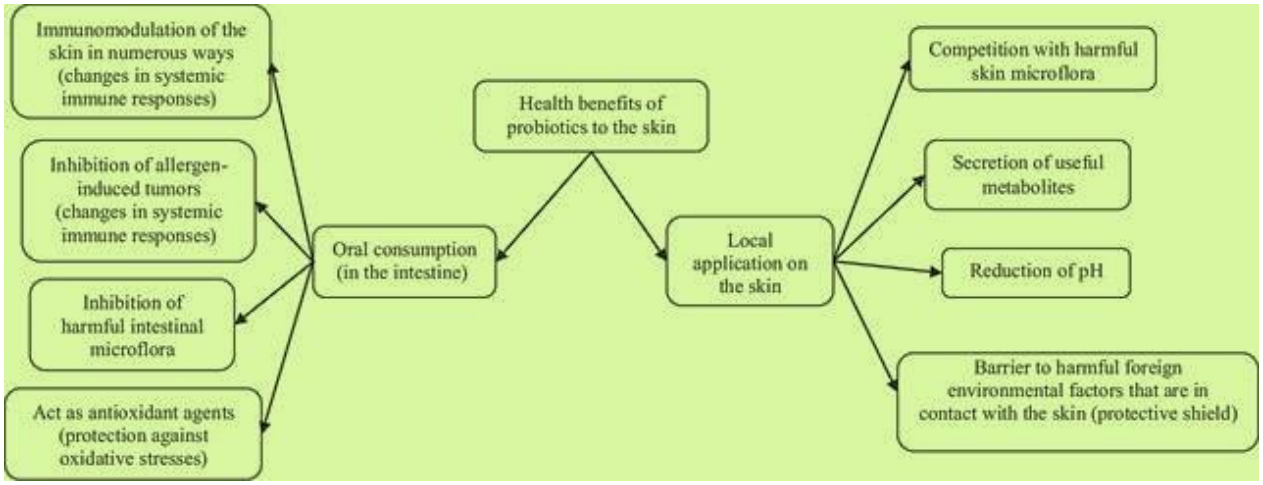


Figure 9. The main mechanisms involved in health benefits of probiotics to the skin [11].

Probiotic bacteria with anti-inflammatory properties have beneficial effects on people suffering from neurogenic skin inflammation or autoimmune skin diseases. Also, probiotics can be helpful for non-healing wounds thanks to the ability to protect the keratinocytes from oxidative stress or to induce skin re-epithelization. Moreover, it has been demonstrated that specific probiotic strains counteract skin aging by reversing photodamage. This finding underlines the potential of probiotics as supporting agents in skin health. However, additional clinical studies are needed to support their routine utilization in medical practice.

Neurogenic Skin Inflammation. Neurogenic skin inflammation is caused by the over-expression of inflammatory cytokines induced by stress conditions and affects hair and skin by reducing hair growth and keratinocyte proliferation, respectively. An *in vivo* study on female C57BL/6 mice demonstrated that *L. reuteri* BM36301 supplementation caused an active hair growth, counteracted the hair follicle regression, and stopped the recruitment of activated macrophages and dendritic cells to the perifollicular zone [157]. In another research, the cell-free extract from *B. longum* was reported to have anti-inflammatory properties and protect skin from physical and chemical aggression using an ex vivo skin explant model [150].

Acne Rosacea, and Acne Vulgaris. Alterations in skin microbiota composition and concurrent bacterial overgrowth in the small intestine are very common features among people with acne rosacea. Indeed, *Propiobacterium acnes* overpopulation has been

documented in acne patients. Conventional therapy to treat acne involves antibiotics targeting *P. acnes*. Interestingly, the enhancement of *Staphylococcus epidermidis* in the skin population has been reported to exclude *P. acnes* from sebaceous hair follicles. The antimicrobial effects of *S. epidermidis* are related to his ability to produce SCFAs known to have direct microbicidal actions against *P. acnes* [158]. Of note, it has been shown that aqueous cell-free extract of *L. brevis* DSM17250 can stimulate the proliferation of *S. epidermidis*; therefore, the supplementation with this probiotic may have indirect antimicrobial effects on skin pathogens [159]. Other probiotic strains have the ability to inhibit *P. acnes* directly. Results from in vitro experiments reported the antimicrobial effects against *P. acnes* of *L. casei* NCFB 161, *L. acidophilus* NCFB 1748, *L. plantarum* DSM 12028, *L. gasseri* NCFB 2233, and *Lactococcus lactis* NCIMB 6681 strains [160]. Also, a bacteriocin produced by *Lactococcus* sp. HY 449 was effective in inhibiting the pathogen growth in vitro and when applied topically in human patch tests [161]. Probiotic supplementation could also be used for the improvement of inflammation, a crucial aspect of acne onset. The probiotic strain *Escherichia coli* Nissle 1917 administered orally to patients with acne and other intestinal-borne dermatoses was able to decrease pro-inflammatory cytokines levels, and normalized immunoglobin-A (IgA) counts and gut function compared to control group. In addition, the skin lesions were significantly smaller or completely recovered [162]. Insulin signaling is a decisive factor in post-adolescence acne vulgaris onset. Insulin, insulin-like growth factor-1 (IGF-1), and defective nuclear transcription factor forkhead box protein O1 (FOXO1) promote acne by stimulating sebaceous lipogenesis and androgen signaling. A recent clinical study reported that consumption of *L. rhamnosus* SP1 for 12 weeks decreased IGF-1 and increased FOXO1 gene expression that, in turn, resulted in alleviation of acne manifestations in adult patients [163].

Psoriasis. Chronic inflammation is an important factor in the development of autoimmune diseases. In particular, T-cells residing in the skin of patients with psoriasis overproduce IL-17 in response to IL-23, causing the production of pro-inflammatory mediators (such as IL-1β, IL-6, IL-8, TNF-α) and chemoattractants from keratinocytes. These signaling molecules further amplify chronic skin inflammation and cause epidermal hyperplasia, the major trait of psoriatic plaques [164]. The utilization of

immunomodulatory probiotic strains could represent a novel therapeutic approach to tackle skin auto-immune diseases. In line with this hypothesis, the therapeutic potential of *L. pentosus* GMNL-77 was evaluated, using a BALB/c mice psoriasis model. The application of this probiotic strain on skin lesions led to a downregulation of pro-inflammatory cytokines, a decrease of the spleen size and differentiation of T-cells, and an alleviation of the erythema and inflammation signs [165]. Moreover, the consumption of *B. infantis* 35624 exerted a decrease in the pro-inflammatory markers IL-6, TNF-α, and serum CRP in psoriasis patients. Finally, in a clinical study exploring the beneficial effects of probiotics for skin lesions in patients with IBD, a decrease of psoriasis skin lesions occurrence was observed in patients that consumed VSL#3 [166].

Atopic Dermatitis. Atopic dermatitis (AD) is the consequence of a Th1/Th2 leukocyte population imbalance that causes an excessive mast cell degranulation and a Th2-mediated allergic response. Phenotypically, this manifests with skin erythema, hemorrhage, and itching that could be caused by both genetic and environmental factors. Most of the researches focusing on AD treatment study two parameters; inflammation and the composition of gut and skin microbiota. Numerous studies on AD have used probiotic strains with anti-inflammatory properties. In work by Kim et al [167]., BALB/c mice were sensitized with whey protein after witch a probiotic mixture of *L. casei, L. plantarum, L. rhamnosus, B. lactis*, and sodium butyrate was administered *per os*. It was shown that probiotic supplementation switched T cell differentiation towards Th1 and Treg populations, and concurrently, the microbiota composition was modified, promoting the reduction of type I hypersensitivity [167]. Similarly, the oral administration of *L. plantarum* IS-10506 decreased the levels of inflammation markers, such as IL-4, IL-17, and interferon-γ (IFN-γ), and enhanced the expression of immunomodulatory factors Forkhead box P3 (Foxp3+) and IL-10 in BALB/c mice and pediatric AD patients. These modifications come with a reduction in the Scoring Atopic Dermatitis Index (SCORAD) [168]. Also, the oral administration of *L. acidophilus* L-92 significantly reduced the Th2-mediated inflammatory responses and SCORAD scores in adult AD patients [169]. Moreover, the consumption of *L. salvarius* LA307 and *L. rhamnosus* LA305 decreased skin inflammation, hyperplasia, immune cell skin infiltration, and hyperkeratosis in hairless SKH-1 mice suggesting their potential

benefits for AD patients [170]. *Lactococcus chungagensis* CAU 28T, showed anti-inflammatory, anti-allergic, and anti-bacterial properties on NC/Nga mice, an inbred AD murine model fed with this strain, lowering the molecular markers of AD and attenuating its clinical manifestations [171]. Similarly, oral administration of tyndallized and freeze-dried *L. rhamnosus* ICDD 3201 on Nc/Nga mice decreased IgE counts, causing the neutralization of AD symptoms [172]. AD skin lesions are often colonized by high loads of *S. aureus*. Therapeutic interventions limiting this pathogenic population result in clinical improvement of skin manifestations. Using a reconstructed human epidermis model in vitro, it has been demonstrated that *L. johnsonii* NCC 533 induced the expression of antimicrobial peptides and inhibited adhesion of *S. aureus* [173]; in addition, the topical application of a lotion containing heat-treated *L. johnsonii* NCC 533 cells improves the lesion skin appearance in *S. aureus*-positive AD patients who participated in an open-label multicenter study. This effect was correlated to the reduction of *S. aureus* population [154].

Aging Skin. Aging skin is characterized by increased pH, oxidative stress, and matrix metalloprotease activity that cause dehydration and wrinkle formation. Results from preclinical and clinical studies indicate that local or oral administration of probiotics could potentially counteract phenotypic skin alterations, restore stratum corneum elasticity, and improve hairs' quality [174]. Environmental factors, such as Ultra-Violet (UV) irradiation, accelerate skin aging. Several studies have demonstrated the regenerative effects of probiotics on UV-induced photodamaged skin. Orally administrated *L. acidophilus* decreased wrinkle formation caused by UVB irradiation in a mouse model [175], while the supplementation with *Bifidobacteria breve* recovered skin hydration and counteracted tight junction and basal membrane photodamage [176]. Also, a mix containing *L. johnsonii* and carotenoids was able to reverse early UV-induced photodamage in healthy women restoring the responses of the skin immune system [177]. Moreover, probiotics have shown the ability to activate endogenous, protective mechanisms. For example, *Vitreoscilla fuliformis* was able to reduce UV-induced oxidative stress *in vitro,* modulating the expression of a mitochondrial oxide dismutase [178]. Park and Bae [179] investigated the anti-senescence and anti-oxidant effects of the fermented *Acanthopanax koreanum* root extract from *L. plantarum* and *B.*

bifidum on human skin fibroblasts showing that the treatment reduced the expression of cell cycle proteins (i.e., p53 and p21Cip1/WAF1), MMP-1, and MMP-3. These effects were mediated by the activation of the mitogen-activated protein kinase (MAPK) signaling pathway induced by the probiotic treatment. Finally, probiotics can be used for skin recovery after cosmetic procedures such as fractional CO_2 laser resurfacing treatments. This technique is used to reduce the signs of aging but is accompanied by a healing process that comprises inflammation, edema, erythema, and crusting. For this reason, the patients need to apply topically, broad-spectrum antibiotics, sunscreen, and hyaluronic acid masks, though none of these alleviate inflammation [180]. In a paper by Zoccali et al. [153] topical probiotic cream led to a statistically significant reduction of postoperative skin inflammation and swelling of the treated areas. The experimental cream containing *S. thermophilus* was administered post-operatively for 2 weeks to 42 consecutive patients who were treated with fractional CO2 laser. The efficacy of the experimental cream was evaluated comparing the rate of post-operative signs vanishing with a control group of 20 patients topically treated with an antibiotic cream and a hyaluronic acid-based cream. The post-operative administration of the probiotic-containing cream induced a quicker reduction of post-operative erythema and swelling when compared to a standard treatment.

New insights could now fundamentally change the impact of probiotics on dermatology. Indeed, an emerging approach to help preventing and treating skin conditions, including the external signs of aging, acne, rosacea, yeast and bacterial infections, psoriasis, and dermatitis, is represented by topical probiotics, as shown by the growing marketplace for topical probiotic formulations available for skin care and antiaging benefits.

In conclusion, topical probiotic formulations are becoming increasingly available for healthy skin care, prevention and treatment of skin diseases, and antiaging benefits, thus representing an emerging area for skin health. The potential benefits of skin probiotics could strongly depend on how each species or strain is selected as the specific mechanisms underlying a specific effect on the healthy or disturbed skin. It appears, therefore, particularly important to stress that it is not possible to generalize the effects that each of them, any association or combination thereof or extracts thereof, has on the skin. The key evidences available from scientific literature as well as registered

patents have been summarized in relation to actual or potential topical applications of probiotics in the field of dermatology by Cinque et al. [181]. A comprehensive model that summarizes the main actions carried out by probiotics in different skin conditions associated with altered microflora, abnormal oxidative stress, disturbed skin barrier, and/or inflammatory/immune skin reactions is shown in **Figure 10**. It is important to note that, for simplicity, the generic term "probiotics" is indicated but it must be implied that the claimed effect should be attributed to specific species or specific strains of probiotics as above specified.

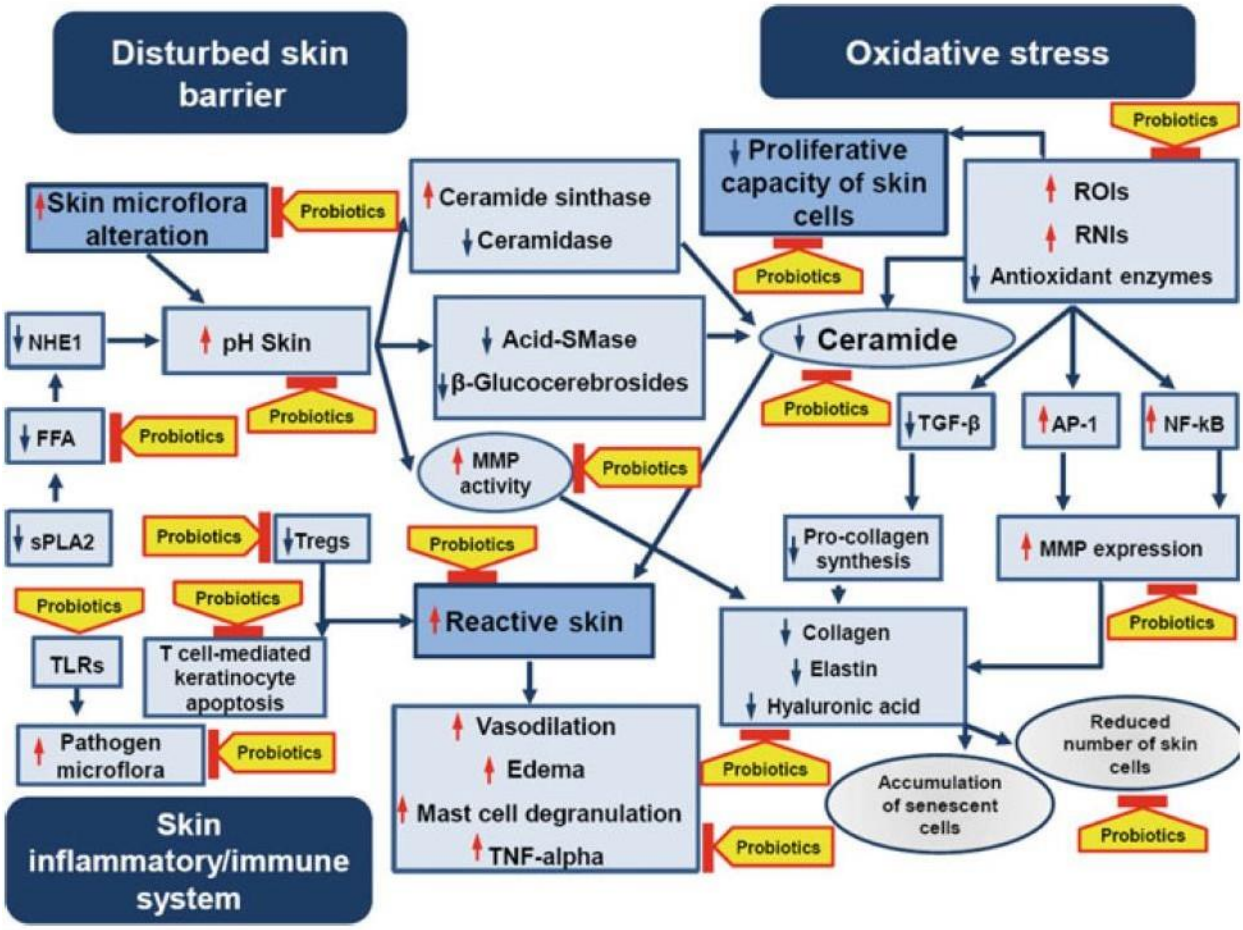


Figure 10. Comprehensive model that summarizes the main actions carried out by probiotics in different skin conditions associated with altered microflora, abnormal oxidative stress, disturbed skin barrier, and/or inflammatory/immune skin reactions [181].

Chronic Conditions and Non-Healing Wounds.

Non-healing wounds represent a major clinical problem in diabetics, elderly, obese, and patients with chronic burn wounds. The topical application of probiotics has

demonstrated to both exclude pathogenic microorganisms and induce re-epithelization and collagen production. Indeed, a patch containing nitric oxide gas-producing *L. fermentum* 7230 and increased collagen synthesis and blood flow to the wounds when applied on infected and ischemic rabbit wounds [182]. In addition, LGG and *L. reuteri* have been reported to induce keratinocyte migration and proliferation [183]. LGG was also able to induce the increase of tight-junction protein levels of claudin 1, occludin, and ZO-1, enhancing the tight-junction barrier function of human primary keratinocytes *in vitro* [184]. The antimicrobial activity of several probiotic strains contributes positively to the management of chronic wounds. In particular, probiotics that adhere to keratin displayed antimicrobial properties against *E. coli*, *P. acnes*, and *Pseudomonas aeruginosa* and inhibited early biofilm formation *in vitro* [185]. Moreover, *S. aureus* colonization could be counteracted by the application of LGG, as well as of skin commensals *S. epidermidis* [186] and *S. hominis* [155].

Future direction. A crucial factor for probiotic efficacy is the appropriate delivery. Probiotics need to be applied topically to have a direct effect on the skin. This approach suffers a major limitation: live probiotics are not compatible with other components of creams, with a consequent rapid organoleptic decline. To overcome this limitation, the researchers have started to focus their attention on the study of the properties of cell-free supernatants (CFS) and inactivated strains, termed as "paraprobiotics" [187]. Some paraprobiotics can exert antimicrobial, anti-inflammatory, and anti-aging actions while being stable in preparations [150,154,173]. In this context, heat-killed *Lactococcus lactis cremonis* H61 protected senescence-accelerated mice from bone loss and aging skin manifestations [188] it is worth notice that sterile CFS and its isolated components make probiotic supplementation available for immunocompromised and immunodeficient subjects, to whom live microorganism consumption is not recommended.

1.4 PROBIOTICS OR PRO-HEALERS THE ROLE OF BENEFICIAL BACTERIA IN TISSUE REPAIR

The probiotics represent potentially a safe therapeutic approach for the treatment of patients affected with cutaneous and intestinal wound healing disorders. The choice of bacterial strains is of particular importance since the effects of probiotics are highly

strain-specific. In addition to the therapeutic potential of topical treatment, the probiotics can promote tissue repair through the gut-skin axis modulation.

1.4.1 The role of probiotics in infections and intestinal wound healing

Close association with epithelial cells makes probiotics effective in antimicrobial treatment of pathogens. Beside the direct inhibition of pathogen growth, probiotics can induce host mucosal defense and tissue repair mechanisms [189].

Direct inhibition of pathogen growth by probiotics

Probiotic can act against pathogens through the production of antimicrobials, the displacement of pathogens from epithelial cells and mucus, and the elimination of pathogens by co-aggregation and quorum quenching. Antimicrobials produced by probiotic strains include organic acids, hydrogen peroxide, diacetyl, reuterin, and bacteriocins. Organic acids, produced by multiple probiotic strains, are primarily responsible for antimicrobial activity against Gram negative pathogens [190]. Hydrogen peroxide produced mainly by lactobacilli, including *Lactobacillus fermentum, L. acidophilus and L. jensenii* were associated with a reduction in the count of fastidious anaerobic Gram-positive bacteria, including *Bacteroides*, *Prevotella*, *Gardnerela* and *Mycoplasma* spp. [191,192]; diacetyl, another metabolic product of lactobacilli exhibits broad-spectrum antimicrobial potential against both Gram negative and Gram positive pathogens [193,194]. Reuterin (3-hydroxypropionaldehyde), a well-known antimicrobial metabolite produced by *L. reuteri*, can specifically inhibit the growth of harmful gut bacteria by oxidizing thiol groups in the target pathogenic microorganism [195]. Notably, reuterin, does not kill beneficial microorganisms, allowing *L. reuteri* to remove gut invaders while keeping normal gut microbiota intact. Reuterin also shows antimicrobial activity against *Staphylococcus,* known as the common chronic wound pathogen [196]. Bacteriocins, another class of probiotic metabolites, are small peptides produced by microorganisms that show a wide range of antimicrobial activity both *in vitro* and *in vivo* [197-199]. The ability of several probiotic strains, such as *Bifidobacterium longum, L. rhamnosus* and *L. delbrueckii* to auto-aggregate is thought to confer their antimicrobial ability to co-aggregate with other microorganisms, including the common wound pathogens *Staphylococcus aureus* and *Candida albicans* [200]. While several *in vitro* studies have shown the potential of probiotics to displace

pathogens from epithelial cells through their aggregation propriety [201], no *in vivo* studies have been conducted. Besides the production of antimicrobial metabolites and co-aggregation, probiotic strains can displace intestinal pathogens from the gut epithelium. This propriety could be attributed to specific surface molecules produced by lactobacilli, e.g., extracellular polysaccharides, which allow *L. paracasei* to competitively adhere to EC cells and displace pathogenic bacteria [202]. Another emerging antimicrobial mechanism of probiotics is the inhibition of pathogen quorum sensing (QS) system, an intercellular communication system used to modify gene expression based on cell-population density, in order to form biofilm and confer virulence [203,204]. Most pathogens, including species commonly found in chronic wounds (e.g., *Pseudomonas aeruginosa* and *S. aureus*), utilize QS for virulence, biofilm formation, and resistance to host defense [204-206]. However, probiotics can interfere with pathogen QS. In particular, *L. plantarum* was shown to inhibit the production of QS signaling molecules (acyl-homoserine-lactone) by *P. aeruginosa*, along with the reduction of biofilm formation [207].

Probiotic effects on epithelial barrier

Besides the direct antimicrobial effects on pathogens, probiotics can improve epithelial barrier function, thus limiting pathogen invasion [208]. Both *in vitro* [209] and *in vivo* [134] studies have shown the ability of probiotics to strength GI barrier by increasing expression and regulating the localization of tight junction (TJ) proteins. Oral administration of *L. reuteri* in newborn piglets led to an increase of occludin, claudin, and zonula occludens 1 (ZO-1) expression in the gut [97], and a clinical study demonstrated that the oral administration of *Lb. plantarum* induced the recruitment of occludins and ZO-1 to the region of TJ [134]. Also, the probiotic mixture VSL#3 supported the suppression of chronic inflammation by improving the epithelial barrier function in murine models of chronic ileitis [210]. In addition to lactobacilli, probiotic bifidobacteria have comparable effects. *B. infantis* increased expression of tight-junction proteins ZO-1 and occludin in human gut epithelia and transepithelial resistance. This was associated with enhanced levels of phospho-ERK and decreased levels of phospho-p38, cell signaling events important for barrier formation [222]. The effects of probiotics on wound healing in the GI tract have been widely examined

through various experimental models, including acetic acid-induced ulcers, full-thickness wounds, and intestinal anastomoses. In these studies, the beneficial effects of lactobacilli were mediated primarily by the activation of epithelial cells, and stimulation of fibroblast proliferation and migration [212,213]. Aside from increasing the epithelium repair, *L. plantarum* was demonstrated to increased collagen synthesis in the intestine [213]. The strengthening of the epidermal barrier by probiotics is closely related to their effects on immune components since the functions of epithelial cells and fibroblasts are tightly regulated by chemokines, cytokines, and growth factors [214]. By the induction of β-defensin, probiotics can affect innate immune components of the intestinal barrier [215]; this molecule is known to promote wound healing, aside from its role in fighting intestinal pathogens [216]. VSL#3 probiotic mixture induces the expression of transforming growth factor β (TGF β) and vascular endothelial growth factor (VEGF) [217], while probiotic formulation containing *Saccharomyces boulardii* induces epidermal growth factor (EGF), its receptor activity (EGFR), and insulin-like growth factor (IGF) [218,219] i*n vivo* murine studies. In addition, *L. rhamnosus* was reported to stimulate hypoxia inducible factor 2α (HIF-2α) *in vivo*, a key regulator of progenitor stem cell recruitment during tissue repair [220,221].

Immunomodulatory properties of probiotics

Probiotics can promote gut health through immune-modulating functions such as the activation of natural killer (NK) cells, dendritic cells (DC), intraepithelial γδ T lymphocytes and macrophages, important effector cells of innate immunity for both skin and GI barrier restoration [210,222]. It has been demonstrated that the immunomodulatory effects of probiotics are species and even strain-specific. For example, *L. sakei* and *L. rhamnosus* stimulated macrophage activity [223], while *L. plantarum* increased phagocytic activity of peritoneal macrophages *in vivo* [224]; these functions were mainly associated with the induction of IL-22, TNFα, IL-6, IL-8, and IL-12 secretion [225]. *L. reuteri* and *L. johnsonii* can also induce secretion of the cytokine IL-22, which is principally produced by intraepithelial T lymphocytes [226,227]. Metabolites of these probiotic strains can bind to and activate the aryl hydrocarbon receptor (AhR) expressed on macrophages and DC, with the subsequent activation of epithelial cells [203,226,228]. TNFα, the most important cytokine in the innate immune

response secreted mainly by monocytes and macrophages, but also by epithelial cells [229]. This cytokine is known to be induced by systemic administration of probiotics; in this regard, *L. casei* induced TNFα production, along with IFNγ and IL-10 in healthy mice when orally administrated [230]. *L. fermentum* treatment also increased the expression of TNFα along with an increase of neutrophil infiltration [231], which can help to fight infection. Production of IL-8, a crucial chemokine for the recruitment of neutrophils, also correlated with lactobacilli treatment [232]. It is well documented that probiotics can also influence the production of cytokines that regulate adaptive immune responses; lactobacilli were shown to induce production of IL-10 and IL-12, which are at the crossroads of innate and adaptive immune responses, leading to expansion of T regulatory lymphocytes (Treg) or Th1 cells in a strain-specific manner [230,233,234]. The ability of the treatment with multiple probiotic strains to activate NK cells has been demonstrated both in *in vitro* and *in vivo* studies [234,235] Given the crucial role of NK cells in fighting pathogens, including the common skin pathogen *S. aureus* [236], further research focusing on the protective role of probiotics in treating both intestinal and cutaneous wound infections are needed.

1.4.2 Probiotics and cutaneous wound healing

In vitro studies

Probiotics are studied as a potential treatment for several dermatologic disorders, such as non-healing wounds [237]. Numerous *in vitro* studies on human keratinocytes showed the protective abilities of probiotic strains towards skin pathogens [183,184,238]. Similar to their effect on the gut epithelium, *L. rhamnosus GG* and *B. longum* have been shown to increase TJ function and expression of claudin 1, ZO-1, and occludin in keratinocytes infected with *S. aureus* [184], suggesting that they can influence TJ function decreasing paracellular permeability and thus preventing pathogen invasion. Moreover, activation of Toll-like Receptor 2 (TLR2) improves the tight barrier function in gut epithelial cells, as well as in keratinocytes [239]. The ability of *B. longum* to modulate TJ function appears to be TLR2 dependent as the increase in TEER and TJ protein levels was abrogated when TLR2 was neutralized or blocked, respectively [184]. Conversely, the effects of the *L. rhamnosus GG* on keratinocytes were TLR2-independent, suggesting that this strain uses an alternative way to improve tight barrier

function. Other probiotic strains such as *L. reuteri* and *L. plantarum* have shown the ability to enhance tight barrier function in primary human keratinocytes. They also increased re-epithelialization, accelerating keratinocyte migration and proliferation [183]. Probiotics can also induce re-epithelialization through the induction of chemokines. For example, *L. rhamnosus* GG increased expression of the chemokine CXCL2 and its receptor CXCR2, stimulating keratinocyte proliferation and migration in a scratch wound assay model *in vitro*. While most of the probiotics have shown to be beneficial for keratinocyte function, it has been reported that *L. fermentum* reduce keratinocyte viability and re-epithelialization [183,184], emphasizing again strain-specific effects. Another method of improving wound healing is the protection against cutaneous wound infections through probiotic antibacterial activities such as inhibition of pathogen growth and reduction of pathogen adhesion. *L. rhamnosus GG* has shown this protective effect, inhibiting *S. aureus* growth in infected keratinocytes, although the exact mechanism has yet to be identified [238]. Additionally, *L. plantarum* supernatant was able to inhibit pathogenic properties of *P. aeruginosa,* a common chronic wound pathogen, by interfering with its quorum sensing system [240]. This cell-free supernatant was capable of reducing bacterial adhesion and biofilm growth through inhibition of elastase, pyocyanin, and rhamnolipids, *P. aeruginosa* virulence factors. Lactobacilli have also shown the ability to inhibit pathogen invasion into keratinocytes by competitive exclusion. *L. reuteri* and *L. rhamnosus GG* can prevent the initial adhesion of *S. aureus* to keratinocytes and displace *S. aureus* already attached to human keratinocytes [238,241]. The specific molecules involved in this mechanism are still unidentified, but they could involve a class of multifunctional bacterial adhesins that can, among other functions, bind to epithelial cells, the moonlight proteins: [242]. An example of a moonlight protein is enolase; the enolase from *L. crispatus* has been shown to bind to laminin and collagen I [243], while enolase from *L. plantarum* can bind to fibronectin, preventing *S. aureus* adhesion to epithelial cells [244]. The lysates, supernatants, and metabolites from probiotic strains have been widely studied *in vitro* and *in vivo,* demonstrating beneficial effects similar to live microorganisms [238,240].

In vivo **studies**

In vivo wound healing studies have mostly been focused on topical application of probiotics showing their ability to improve tissue repair and reduce the bacterial load in rodent wound models [207,245,246]. Topical application of *L. plantarum* inhibited wound colonization by *P. aeruginosa* in a burn mouse model by clearing *Pseudomonas* from the skin, liver, and spleen, through enhanced phagocytosis [207]. *L. plantarum* inhibited pathogen colonization also in human burn wounds infected with *P. aeruginosa*, *S. aureus*, and *S. epidermidis* [149], decreasing bacterial load and promoting wound healing. One potential mechanism underlying *L. plantarum* anti-pathogenic properties is this ability to induce IL-12 secretion, which activates cytotoxic T cells and NK cells to secrete IFNγ [247]. Topical *L. plantarum* also improved wound healing in human chronic venous ulcers infected with *S. aureus* and *P. aeruginosa*, reducing bacterial load and inducing granulation tissue formation. *L. plantarum* is could inhibit pathogen colonization by regulating IL-8 levels and modulating the entry and activity of PMNs migrating from peripheral blood to the ulcer. Indeed, the polymorphonuclear cells (PMN) isolated from the ulcer bed showed increased IL-8 production and decreased apoptosis and necrosis after the treatment with *L. plantarum* [151].

Beneficial effects of cutaneous probiotic strains

Aside from lactobacilli and bifidobacteria, other probiotics, commonly associated with healthy skin microbiome, have shown beneficial effects on the cutaneous repair. *S. thermophilus* is a LAB that, through its sphingomyelinase activity, can increase the level of ceramides and phosphorylcholine in keratinocytes [144]. Ceramides play a crucial role in developing extracellular lipid bilayers and therefore improving the lipid barrier of the skin [248]; the topical application of *S. thermophilus* increased ceramide levels in the stratum corneum of healthy skin [144]. Reduced levels of ceramide have been associated with barrier dysfunction in the epidermis, including lack of protection against bacteria [249]. Consequently, probiotic treatments able to increase ceramide levels have the potential to improve the skin barrier function during the wound healing process. Another widely-studied skin commensal probiotic is *S. epidermis*. This strain secretes Esp, an extracellular serine protease that can inhibit colonization by *S. aureus* through many mechanisms. Esp can inhibit *S. aureus* biofilm

formation by degrading biofilm matrix and cleaving host receptor proteins important for *S. aureus* adhesion and infection, such as Protein A, fibronectin, fibrinogen, and vitronectin [250]. Although *S. epidermidis* has been implicated as a beneficial microorganism for the management of inflammation associated with atopic dermatitis and acne, its application in the context of wound healing need to be further investigated [25]. In addition to l *Staphylococcus*, other commensal bacteria such as *Vitreoscilla filiformis* have shown the ability to reduce cutaneous inflammation through stimulation of regulatory T cells and skin dendritic cells [24].

Probiotic effects through gut-skin or gut-brain-skin axes

Besides topical effects on the cutaneous barrier, gut probiotics may modulate skin wound healing through effects on systemic immunity, enhancing nutrient absorption, and modulating gut-brain-skin axis. Probiotics can produce neuroactive molecules and modulate the secretory activity of enteroendocrine cells (EEC) in the gut mucosa, such as oxytocin, leading to the release of neuromodulators with the potential to improve tissue repair. Gut probiotics can also contribute to the activation of innate and adaptive immune responses stimulating the recruitment of polymorphonuclear lymphocytes (PMN) to the injured tissue and T-cell priming in skin lymph nodes. Moreover, gut probiotics increase the intestinal absorption of nutrients important for wound healing. Several studies have demonstrated an association between the gut microbiota, the immune system, and skin health [251-254]. The influence of the gut microbiome on systemic and local immunity explains why gastrointestinal diseases such as IBD and vascular disorders also have cutaneous manifestations [255]. Oral assumption of probiotics can modulate local as well as systemic immune response [175,256,257]. Oral supplementation with probiotics increased the frequency of Treg cells in skin lymph nodes while decreasing inflammation in a murine model of skin allergy [251]. Some probiotic strains have the potential to combat skin inflammation when administrated systemically modifying the composition of gut microbiota. Consumption of probiotics such as *L. reuteri* led to an upregulation of the anti-inflammatory cytokine IL-10, which induced Foxp3+ Treg lymphocytes to decrease tissue damage at the wound edge [258] and, in turn, down-regulate the pro-inflammatory cytokine IL-17A [259,260]. The beneficial systemic effect of gut bacteria could be

achieved by direct translocation of bacterial antigens to peripheral circulation via dendritic cells, instead of their transport to local lymph nodes [261]. Further studies are needed to reveal the exact processes behind the immune effects elicited by gut bacteria influencing cutaneous wound healing.

Aside from affecting the host immune system, systemic effects of gut microbiota and orally introduced probiotics could be mediated by their ability to improve the absorption of nutrients crucial for skin wound healing, such as vitamins, minerals, and cofactors for enzymes involved in tissue repair [262-264]. Moreover, the synthesis of short-chain fatty acid by probiotics increases mineral solubility by decreasing luminal pH [265]. In addition, several lactobacilli species, such as *L. coryniformis* and *L. rossiae*, can produce vitamin B12 known to be beneficial for wound healing [266,267], and *L. reuteri* and *Lb. acidophilus* can increase the absorption of dietary vitamin D and E, which are important for wound healing [268-270].

It has been reported that gut microbiota can also influence the central nervous system [271]. Furthermore, intestinal bacteria and their metabolites can interact with neuroendocrine pathways that modify stress-related responses in the skin through the gut-brain-skin axis [259,272]. Probiotics can offer a potential therapeutic option that could beneficially modify this axis, thereby improving systemic health for patients affected with cutaneous and wound healing disorders. *L. reuteri*-induced oxytocin is the most widely studied example of probiotic influence on the gut-brain-skin axis [257]. In this regard, dietary supplementation with *L. reuteri* lysate was able to increase systemic oxytocin levels, increasing oxytocin-producing cells in the hypothalamus of mice. This treatment resulted, also, in lower blood levels of the stress hormone corticosterone and accelerated epidermal wound closure. Oxytocin receptors present on both fibroblasts and keratinocytes might probably influence this process [273]. It is also known that oxytocin reinforces host immunity by increasing CD25 and IFN-γ expression in thymic and peripheral lymphocytes [274,275], thus it may enhance wound healing through improving host immune functions [258,259]. Other gut lactobacilli were also shown to produce neuroactive molecules, such as catecholamines and gamma-aminobutyric acid (GABA) [276,277]. Moreover, a probiotic formulation containing *L. helveticus* and *B. longum* was able to reduce stress response in humans and rats, and this effect was

associated with reduced levels of urinary cortisol [278] known to impair wound healing [279].

These data suggest a strong link between the gut microbiome and cutaneous health through the multiple interactions within the gut-brain-skin axis. However, these links are not fully investigated yet, and though a therapeutic potential of probiotic bacteria for wound healing disorders exists, more definitive studies are needed.

2. RATIONALE AND AIM

The doctoral project aimed to investigate the *in vitro* effects of selected strains of probiotics on two different epithelial models, to study their ability to influence tissue repair.

MODEL #1: SCRATCHED KERATINOCYTE MONOLAYER

As above stated, the use of probiotic formulations would thus represent a valid alternative approach to overcome the existing problems of actual wound therapy approaches, including the high costs, the long manufacturing times, and the increase in antibiotic resistance. However, further studies are needed both to identify probiotics or any combinations of them in terms of therapeutic efficacy and to fully define the underlying mechanisms. In this regard, it seems quite surprising that, no literature reports are available about possible involvement in the pro-healing mechanisms activated by some probiotics, of nitric oxide (NO), one of the most important players in the regulation of the wound repair process [280-283]. As yet mentioned in the Introduction's section, the levels of NO metabolites are shown to correlate with the healing trajectory indicating the tendency of recovery or exacerbation [284]. The application of exogenous gaseous NO or the NOS2 stimulator [285], the transfer of the NOS2 gene [286], and the systemic supply of the NOS substrate, i.e., arginine [287], the employment of NO donor systems [288] are all approaches capable of elevating the local NO concentration, and consequently, promoting wound recovery. On the other hand, a blockade or knockout of NOS2 impaired wound healing [289-292]. Given the crucial role played by NOS2/NO system in the wound repair process, after comparing the ability of the soluble fraction from lysates of seven different probiotic strains to affect the re-epithelialization process in vitro, the involvement of NOS2 expression and activity in the mechanisms underlying the probiotic effects are firstly investigated in the model of scratched keratinocyte monolayer. With this purpose, it was first analyzed the effect of the soluble fraction of seven selected strains of probiotics on a wound healing model using the HaCaT cell line. Screening of bacterial strains was carried out to identify those able to accelerate the repair process; subsequently the effects of these strains on the two main processes involved in wound healing were evaluated: cell proliferation and migration. The involvement of NO was analyzed in the acceleration

process induced by the different bacterial strains evaluating, in particular, the expression of NOS2.

MODEL #2: INTESTINAL EPITHELIAL BARRIER IN VITRO

The group of Prof. Cifone has previously reported the results obtained comparing the US-made VSL#3 (now named VIVOMIXX) *versus* the Italy-made VSL#3. The two products, even though sold under the same brand VSL#3, differently influence *in vitro* tumor cell lines as well as repair process of scratched intestinal epithelial cell monolayer [293,294].

Since a recent paper using animal models of IBD has suggested that the "new" Italy-made formulation failed to reduce gut inflammation and worsened intestinal permeability, the epithelial barrier model of human colon cancer cells CaCo-2 was chosen as another *in vitro* model to investigate some mechanisms which may contribute to explain what observed in the animals and *ex vivo* in humans [295,296]. CaCo-2 are indeed widely used as an optimum *in vitro* model for studies on intestinal barrier functions [297-299] due to their ability to spontaneously differentiate into a polarized monolayer when grown on porous inserts. Under these conditions, CaCo-2 close up and tighten becoming similar to the intestinal barrier composed by enterocytes with microvilli (brush border) on the apical side and a basolateral side, and express TJ (Tight Junction) proteins, similar to the small intestine [300,301]. In particular, the effects of a probiotic formulation (VIVOMIXX), composed by eight probiotic strains were analyzed on trans-epithelial electrical resistance (TEER), dextran flux, and expression TJ proteins i.e. zonulin-1 (ZO-1) and occludin, in the absence or presence of a heat stress-related damage of cell monolayer.

3. MATERIALS AND METHODS

3.1. PREPARATION OF BACTERIAL SAMPLES FOR CELL TREATMENTS

B. longum BL-04, *B. infantis* Bi-07, *B. breve* BB-03, *S. thermophilus* St-21, *L. bulgaricus* TR-160, *L. plantarum* Lp-115, and *L. acidophilus* LA-14 in a pure lyophilized form were kindly provided by Prof. Claudio De Simone, MD. For bacterial lysate soluble fraction preparations, stocks of 10^8 CFU/g of each lyophilized probiotic resuspended in Phosphate Buffered Saline (PBS, Euro Clone, West York, UK) were centrifuged at 8,600×*g*, washed twice, resuspended in 10 mL of PBS and sonicated (30 min, alternating 10 s of sonication and 10 s of pause) using a Vibracell sonicator (Sonic and Materials, Danbury, CT, USA). Bacterial cell disruption was verified by measuring the absorbance of the sample at 590 nm (Eppendorf Hamburg, Germany) before and after every sonication step. The samples were then centrifuged at 17,949×*g* and the supernatants filtered using a 0.22-µm-pore filter (Corning Incorporated, Corning, NY, USA) to remove any whole bacteria remaining. Total protein content was determined by DC Protein Assay (BioRad, Hercules, CA) using bovine serum albumin (BSA, Sigma Aldrich, Saint Louis, MO, USA) as standard. Based on our experience, for *in vitro* experiments, the bacterial soluble fraction was added to cell cultures for different time intervals at 50 µg protein/mL as final concentration.

The probiotic formulation Vivomixx (4.4 g sachet ,450 billion bacteria*)* contains in certain proportions the strains*:* *Streptococcus thermophilus* DSM24731, bifidobacteria (*B. longum* DSM24736, *B. breve* DSM24732, *B. infantis* DSM24737), lactobacilli (*L. acidophilus* DSM24735, *L. plantarum* DSM24730, *L. paracasei* DSM24733, *L. debrueckii* subsp. *bulgaricus* DSM24734). The product was always handled according to the manufacturer's instructions and the sachet once opened was utilized immediately and then discarded. For bacterial sample preparation, stocks of 1 g of Vivomixx formulation were suspended in 10 ml of DMEM supplemented with 1% non-essential amino-acid, 1 mM sodium pyruvate and 2 mM L-glutamine (Euro Clone, West York, UK). For the cell treatment, bacterial final concentration of 10^8 CFU/ml was used.

3.2. CELL LINES AND CULTURE CONDITIONS

The spontaneously immortalized human keratinocyte HaCaT cell line was purchased from Cell Lines Service GmbH (Eppelheim, Germany). HaCaT cells were cultured in DMEM supplemented with 10% (*v*/*v*) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Euro Clone, West York, UK). Culture conditions were kept constant at 37 °C in a 5% CO_2 humidified atmosphere. Under these culture conditions, HaCaT cells remain proliferative and undifferentiated. After reaching 80% confluence, cells were seeded, as below specified, into a sterile tissue culture 6-well plate or 12-well plate (Becton Dickinson, San Jose, CA, USA) at 18,000 cells/cm^2. To evaluate the effect of the soluble fraction of bacterial lysate on cell viability and proliferation, the cells were cultured in 12-well plate with bacterial samples at 50 µg protein/mL for different time intervals (20–48 h) after which were washed with PBS, centrifuged for 10 min at 400×*g* and the pellets incubated with a 0.04% Trypan blue (Euro Clone, West York, UK) solution for 5 min to analyse cell number and viability. Not-treated cells were also analyzed and served as controls. Cells were transferred to a Bürker counting chamber and counted by microscopy (Eclipse 50i, Nikon Corporation, Japan). To evaluate the effect of Vivomixx formulation on an epithelial differentiated cell monolayer, the human colon adenocarcinoma cell line, CaCo-2 was used, purchased from Sigma-Aldrich (St. Louis, MO, USA) and cultured as previously described (Primavera *et al.*, 2018). Briefly, cells were grown in the DMEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid, 1 mM sodium pyruvate and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium), in a humidified incubator 5% CO2, 95% air atmosphere at 37° C. The cell culture medium was replaced every 2 days until cells reached 60–70% confluence and then harvested using a trypsin-EDTA solution to detach them from the bottom of the flask. CaCo-2 cells with a passage number of 18 to 20 were used for all experiments. Cell viability was analyzed by the trypan blue exclusion staining (0.04% final concentration for 5 minutes). Cells were counted in a Bürker chamber by microscopy (Eclipse 50i, Nikon Corporation, Japan). A set of experiments was performed to also evaluate the effects of a pre-treatment of CaCo-2 monolayer for 4 hours with the Vivomixx mixture at 10^8 CFU/ml on ZO-1 and occludin expression levels in heat-stressed CaCo-2 monolayers (40°C for 10 minutes).

3.3. IN VITRO WOUND HEALING MODEL

As previously described [280], for the *in vitro* wound healing assay, HaCaT cells were cultured in 6-well microplates under normal culture conditions to reach ~90% confluence, then DMEM was removed from the wells and cell monolayers were scratched using a 200 µL pipet tip to create a uniform cell-free wound area with reproducible width of wounding. Debris was removed from the culture by gently washing with sterile PBS. Cell cultures were then incubated with fresh medium at 37°C in a 5% CO_2 humidified atmosphere in the presence or absence of bacterial soluble fraction at 50 µg protein/mL as final concentration. Where indicated, cells were pre-treated for 15 min with 20 µM aminoguanidine (AG), a selective NOS2 inhibitor (Sigma Aldrich, St. Louis, MO, USA). Cell migration was monitored by microscopy using an inverted light microscope (Eclipse TS 100, Nikon) and images were captured (10× magnification) at different time points after the injury (0–45 h). The experiments were conducted in duplicate with at least three fields evaluated for each condition. To calculating the % wound closure, the images acquired were analyzed quantitatively using the TScratch software [302]. Quantification of relative wound closure was performed according to the equation:

$$\%\ \text{Relative Re-epithelialization} = \frac{[\%\ \text{scratched area at T0} - \%\ \text{scratched area at Tn}]\ (\times 100)}{[\%\ \text{scratched area at T0}]}$$

where Tn is a specific time point (h) after the scratching.

3.4. WESTERN BLOT ANALYSIS

For Western blot analyses, scratched monolayers of untreated and bacterial soluble fraction-treated HaCaT cells for 28 h, and CaCo-2 pellets collected after incubation with or without Vivomixx, were harvested, washed in PBS and lysed in RIPA buffer (Merck KGaA, Darmstadt, Germany) containing a protease inhibitor mixture (Sigma Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 600xg for 20 min at 4°C and the supernatants were quantified for protein content using the BCA protein assay kit (Pierce, Rockford, USA). using BSA as standard. 25 µg of proteins were mixed with sample buffer, boiled for 5 min at 100 °C and separated by 10% or 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto 0.45 µm

nitrocellulose membrane sheets (BioRad) for 1 h at 4 °C at 70V using a Mini Trans-Blot Cell apparatus (BioRad). Membranes were blocked with 5% not-fat dry milk for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies. In particular for HaCaT homogenates, rabbit polyclonal antibody anti-NOS2 1:500 (Cell Signalling Technology, CA, USA) or with goat anti-β-actin antibody 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody at 1:5000 for anti-NOS2 antibody and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG secondary antibody at 1:5000 for anti-β-actin antibody (Millipore EMD, Darmstadt, Germany) were used. For CaCo-2 homogenates, primary antibodies anti-occludin, anti-ZO-1 and anti-GAPDH (Origene, 9620 Medical Center Drive Suite 200 Rockville, MD, USA) were used. As secondary antibody an anti-rabbit IgG (for occludin and ZO-1 reactivity detection) and an anti-mouse IgG (Millipore, Burlington, Massachusetts, United States) for GAPDH reactivity detection were used, respectively. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech), according to the manufacturer's instructions. Band relative densities were determined using a chemiluminescence documentation system ALLIANCE (UVITEC, Cambridge UK), and values were given as relative units. Immunoblot data of NOS-2 were normalized to β-actin protein levels, relative ZO-1 and occludin band intensities were normalized to relative GAPDH bands.

3.5. NITRITE LEVEL ASSAY

The enzymatic activity of NOS2 was evaluated by measuring nitrite levels using nitrate reductase and Griess reaction through a colorimetric assay (Nitrite Assay kit-Sigma-Aldrich Co., Milan, Italy). Supernatants (80 μL) of the scratched monolayer of untreated and bacterial soluble fraction-treated HaCaT cells for 28 h were applied to a microtiter plate well for nitrite analysis according to the manufacturer's instructions. The absorbance was measured by spectrophotometric reading at 550 nm using a microplate reader (Bio-Rad Hercules). The nitrite content of each sample was evaluated with a standard curve obtained by linear regression made with sodium nitrite and expressed in μg/mL. Each sample was assayed in duplicate.

3.6 TRANS-EPITHELIAL ELECTRICAL RESISTANCE (TEER)

Epithelial permeability was evaluated by Trans-Epithelial Electrical Resistance (TEER) using a Millicell® ERS-2 (Electrical Resistance System, Millipore, MA, USA) equipped with STX01 electrode (World Precision Instruments, Sarasota, FL) on CaCo-2 differentiated monolayers. CaCo-2 cells were seeded at a density of $4.5x10^5$ cells/cm^2 on polycarbonate inserts in 12 well plates transwell chambers (12 mm diameter, 0.4 µm pore size) (Falcon, One Riverfront Plaza, NY, USA). Each insert was placed in a well in a 12-well plate with 1 ml of media in the apical chamber and 1.5 ml media in the basolateral chamber. Differentiated CaCo-2 monolayers were used at 18-21 days old. The CaCo-2 monolayers were prepared the day before the TEER assay by removing the media and adding DMEM with 1% non-essential amino-acids (without FBS and antibiotics) to ensure no damage to the bacterial cells. Wells containing only medium were used as blank controls. TEER measurements of each well have been registered at different incubation times (0-6 h) with or without addition of probiotic product at 10^8 CFU/ml as final concentration. A set of experiments was performed to also evaluate the effects of a pre-treatment of CaCo-2 monolayer for 4 hours with the VSL#3 mixture at 10^8 CFU/ml to TEER impairment induced by heat stress exposure (40 °C for 10 minutes). The CaCo-2 monolayer TEER values were calculated subtracting the blank resistance (background electrical resistance from an insert without cells including filter and medium) and multiplying the result by the effective growth area of the membrane (1.12 cm^2). TEER values at >500 Ω/cm^2 indicated the successful establishment of the *in vitro* intestinal epithelial barrier model. Results are expressed as a percentage of initial value (baseline).

3.7 PARACELLULAR PERMEABILITY ASSAY

Epithelial permeability across polarized CaCo-2 monolayers was assessed by measuring the flux of the fluorescein isothiocyanate (FITC)-labelled dextran FD4 (1 mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) from the apical chambers to basolateral chambers. FD4 solution was added to the apical chamber, for 2 hours at 37°C in sterile conditions. Fluorescence intensity in the basolateral compartment was measured by fluorometer (Perkin Helmer Victor X4) at excitation and emission

wavelength of 492 nm and 520 nm, respectively. Known concentrations of FITC-dextran FD4 were used to perform a standard curve obtained by serial dilutions of FD4. A set of experiments was performed to also compare the effects of a pre-treatment of CaCo-2 monolayer for 4 hours with the USA-made formulation at 10^8 CFU/ml to dextran flux increase induced by heat stress exposure (40 °C for 10 minutes).

3.8. STATISTICAL ANALYSIS

Data were analyzed using Prism 6.0 GraphPad Software, San Diego, Ca. Results are expressed as means of two or three experiments in duplicate ± SEM. For the comparison of groups, a one or two-way ANOVA test followed by Bonferroni or Dunnett *post hoc* test were used, as specified below. For comparison between two means, Student's unpaired t-test was used. The statistical correlation was calculated through Pearson's test. Results were considered significant if $P < 0.05$. For statistical analysis of data sets * or $^{\#}$ as $P < 0.05$, ** or $^{\#\#}$ as $P < 0.01$, *** or $^{\#\#\#}$ as $P < 0.001$, and **** or $^{\#\#\#\#}$ as $P < 0.0001$, were used throughout the manuscript.

4. RESULTS ON MODEL #1: SCRATCHED KERATINOCYTE MONOLAYER

4.1 EFFECTS OF THE SOLUBLE FRACTION FROM BACTERIAL LYSATES ON HACAT VIABILITY AND ON SCRATCHED MONOLAYER WOUND HEALING.

At all the time intervals of incubation with bacterial soluble fraction at 50 µg protein/mL, no significant influence on the cell viability or basal proliferation level compared to the untreated cells was registered, being in all experimental conditions cell viability >90%.

The ability of the soluble fraction from bacterial lysates to influence re-epithelialization was assessed on an *in vitro* artificial wound model. The rate of scratched monolayer closure in the absence or presence of 50 µg/mL of each bacterial sample was evaluated by observing the re-population of the area between the wound edges at different time points after the lesion. To quantitatively analyze the effects of the bacterial soluble fraction on the closure of the wounded area, images obtained by an

inverted light microscope were acquired at different time points after scratching and converted to % closure by using a mathematical system calculating automatically the portion of the area occupied by the cells. In all experiments, the scratched monolayers of control cells (untreated) were closed at 36–42 h. The percentages of re-epithelialization at 20 and 28 h in the presence or absence of bacterial samples were compared to the untreated monolayers. The results expressed as relative re-epithelialization percentages (mean ± SEM from three independent experiments in duplicate), as well as representative images from microscopic observations of scratched monolayers, are shown in **Figure 11A and B**, respectively.

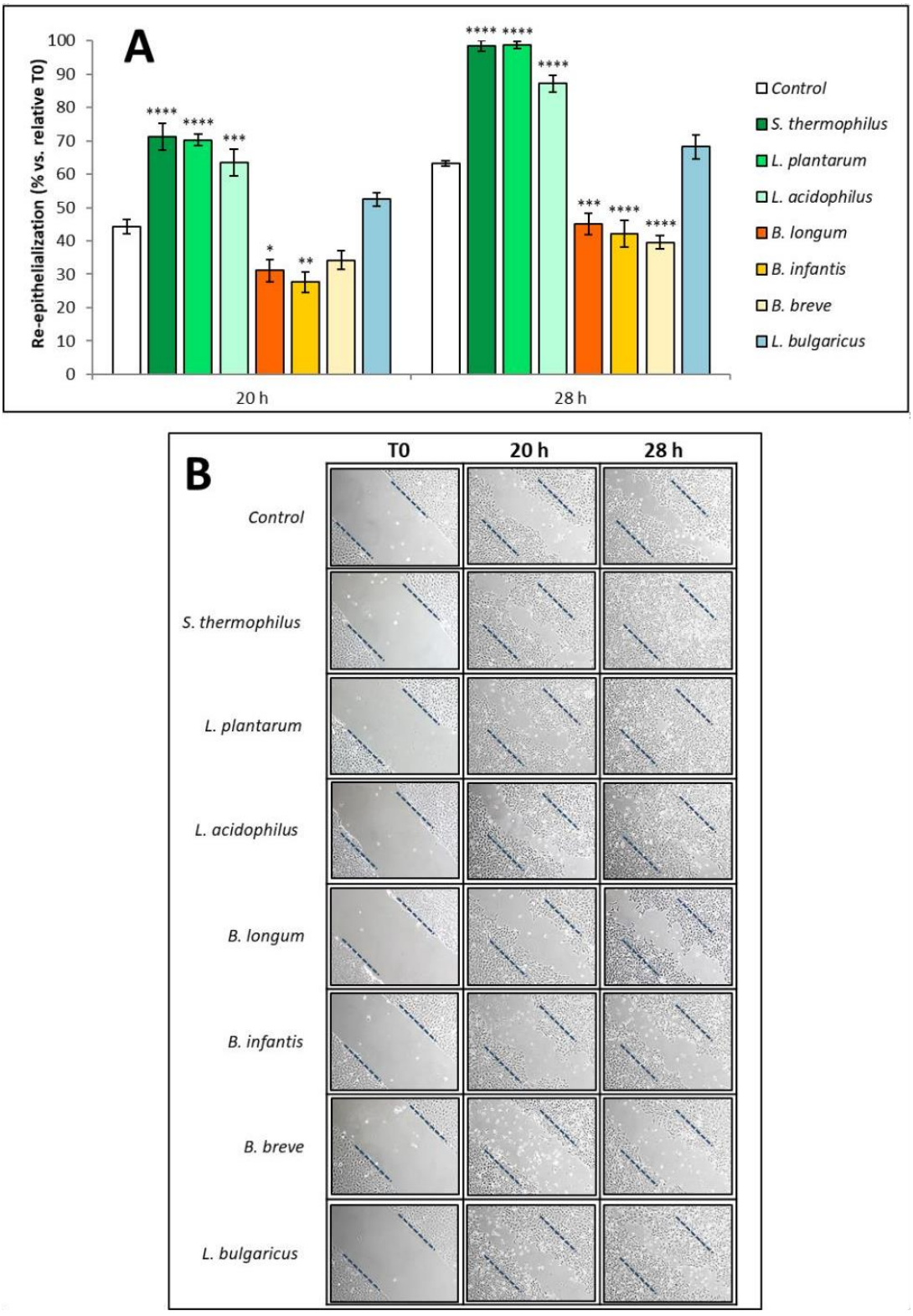


Figure 11. Effect of the soluble fraction of bacterial lysates on re-epithelialization of HaCaT cell line scratched monolayers at different time points. (**A**) Effect of bacterial samples (50 µg/mL) on the wound closure rate (% vs. relative T0) of scratched monolayers at 20 and 28 h. Data are expressed as mean ± SEM of three independent experiments in duplicate. For comparative analysis of groups of data, a two-way analysis of variance (ANOVA) with post hoc Dunnett test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. control (untreated). (**B**)

Representative images of HaCaT monolayer re-epithelialization in the absence (control) or presence of bacterial samples (50 µg/mL) at 20 and 28 h after injury (10× magnification).

The treatment with the soluble fraction from lysates of *S. thermophilus, L. plantarum,* or *L. acidophilus* significantly accelerated the rate of monolayer repair process respect to untreated control at both 20 and 28 h from injury. On the contrary, the treatment with *B. longum, B. infantis,* and *B. breve* samples significantly delayed the monolayer repair process compared to untreated control being the effects more statistically relevant at 28 h. On the other hand, *L. bulgaricus* sample didn't appear to significantly influence the wound closure rate at both observation times when compared to control.

4.2 EFFECT OF THE SOLUBLE FRACTION FROM BACTERIAL LYSATES ON NOS2 EXPRESSION.

To investigate the potential involvement of NOS2 in the above-reported effects of the soluble fraction from bacterial lysates on HaCaT monolayer repair process, we analyzed by western blot the NOS2 protein levels in monolayers after 28 h from scratch in the absence or presence of bacterial samples at 50 µg/mL. The values obtained following the densitometric analysis of NOS2 bands were normalized versus β-actin. Data expressed as mean ± SEM from three independent experiments in duplicate together with a representative image of immunoblotting for NOS2 are shown in **Figure 12A and B**, respectively.

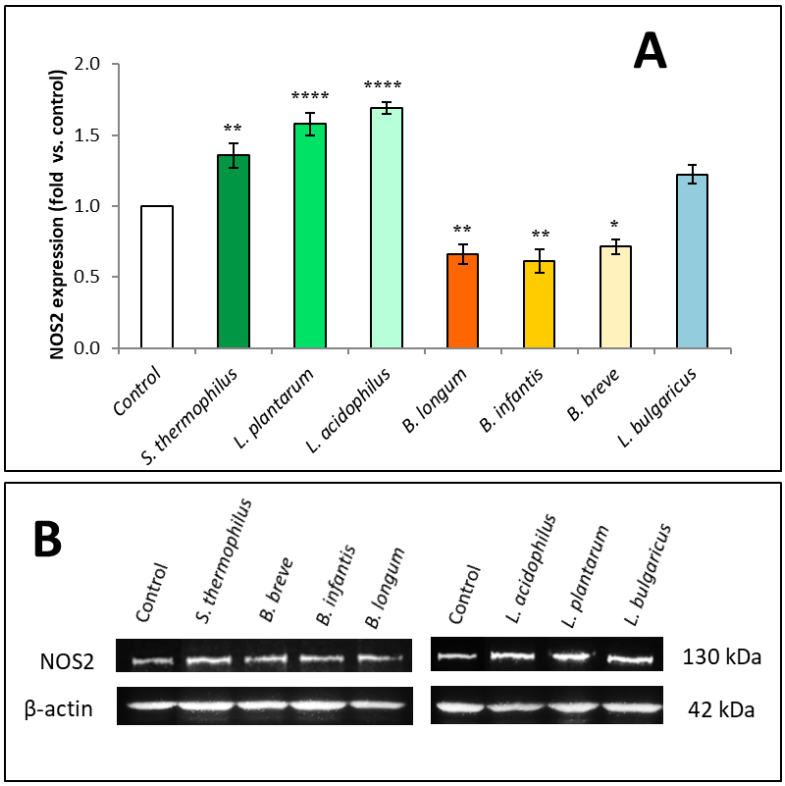


Figure 12. Influence of the soluble fraction from probiotic lysates on NOS2 protein levels in scratched HaCaT monolayers. Immunoblotting assay for NOS2 was performed on scratched monolayers treated for 28 h with 50 µg/mL of probiotic soluble fraction. Following densitometric analysis, obtained values were normalized vs. β-actin and compared with untreated controls. (**A**) Results of densitometry expressed as ratio NOS2/β-actin (fold vs. control). Data are from three independent experiments in duplicate and values are expressed as mean ± SEM. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Dunnett test was used * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ vs. control (untreated). (**B**) A representative image of immunoblotting for NOS2 is shown.

In line with their ability to accelerate monolayer re-epithelialization, the results indicated that the treatment with the soluble fraction from *S. thermophilus*, *L. plantarum*, and *L. acidophilus* lysates led to a marked and significant upregulation of NOS2 protein expression vs. control cells. The most relevant effect could be observed with *L. acidophilus* and *L. plantarum* ($P < 0.0001$ vs. control). *S. thermophilus* sample was also able to significantly increase NOS2 protein expression level even if with statistically lower efficacy ($P < 0.01$). On the other hand, *L. bulgaricus* sample, which did not significantly affect monolayer wound repair, was also unable to modulate NOS2 expression compared to control. Of note, HaCaT monolayer exposed to *B. longum*, *B. infantis*, and *B. breve* samples, all strains able to inhibit the wound closure rate, showed

significantly lower NOS2 protein levels compared to untreated condition ($P < 0.01$ with *B. longum* or *B. infantis*; $P < 0.05$ for *B. breve)*.

4.3 EFFECT OF THE SOLUBLE FRACTION FROM BACTERIAL LYSATES ON NITRITE LEVELS.

The ability of the bacterial samples to regulate NOS2 activity was analyzed by measuring nitrite levels in supernatants of scratched HaCaT monolayers pre-treated with or without the selective NOS2 inhibitor, AG (**Figure 13A**). Pre-treatment with AG prevented the increase of nitrite generation due to NOS activity induced by the scratch of control monolayer ($P < 0.05$), thus confirming previous findings [303]. The treatment with *S. thermophilus, L. plantarum,* and *L. acidophilus* samples, able to up-modulate NOS2 expression, induced a significant increase of nitrite levels in culture medium *versus* control, even if at different extent (*S. thermophilus*, $P < 0.01$; *L. plantarum*, $P < 0.0001$; *L. acidophilus* $P < 0.05$*)*. Of note, the stimulatory effect of *S. thermophilus*, *L. acidophilus,* and *L. plantarum* samples on nitrite generation was totally o partially prevented by pre-treatment with AG, thus supporting the ability of these probiotics to induce NOS2 expression and activity in our experimental conditions. On the contrary, *B. longum*, *B. infantis*, or *B. breve* samples induced a significant decrease of nitrite levels respect to control, confirming their inhibitory effect on NOS2 expression at a similar extent (*B. longum* $P < 0.01$*; B. infantis* $P < 0.05$*; B. breve* $P < 0.01$*)*. In accordance with the results of experiments on NOS2 expression, pre-treatment with AG did not significantly influence nitrite levels in cell cultures treated with the soluble fraction from *B. longum*, *B. infantis*, and *B. breve* lysates as compared to the monolayers treated with bacterial samples alone. The treatment with *L. bulgaricus* sample did not significantly influence nitrite levels compared to untreated cultures, in line with the above results on NOS2 expression and, like control condition, AG pre-treatment significantly decreased nitrite amounts as compared to the relative sample without AG. Using Pearson's test, a significant linear positive correlation resulted between NOS2 expression (fold vs.

control) and nitrite level (fold vs. relative T0) ($r = 0.889$; $P < 0.0001$) (**Figure 13B**).

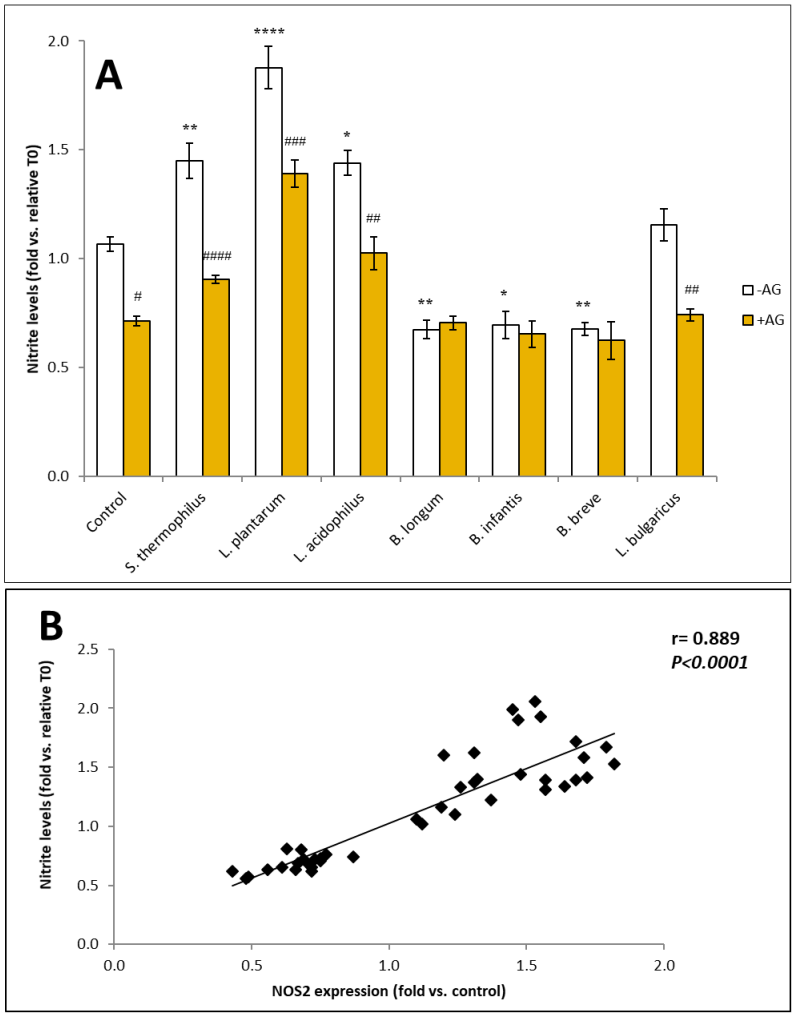


Figure 13. Nitrite levels in HaCaT scratched monolayers' medium in the presence or absence of NOS2 inhibitor AG. (**A**) HaCaT scratched monolayers were incubated with or without 20 µM AG for 15 min before bacterial soluble fraction treatment. After 28 h, nitrite levels in the culture medium were analyzed by Griess reagent. Data shown are expressed as mean ± SEM of three independent experiments in duplicate. For comparative analysis of groups of data, a two-way analysis of variance (ANOVA) with post hoc Bonferroni test was used. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ vs. control (untreated). $^{\#} P < 0.05$, $^{\#\#} P < 0.01$, $^{\#\#\#} P < 0.001$, $^{\#\#\#\#} P < 0.0001$ vs. each relative culture without AG. (**B**) Pearson's test analysis of the correlation between NOS2 expression (fold vs. control) and nitrite levels (fold vs. relative T0).

4.4 EFFECT OF AMINOGUANIDINE ON THE RE-EPITHELIALIZATION OF HACAT SCRATCHED MONOLAYERS TREATED WITH *S. thermophilus, L. plantarum,* and *L. acidophilus.*

The effect of NOS2-inhibitor AG on the rate of wound closure was also evaluated on the ability of *S. thermophilus, L. plantarum*, and *L. acidophilus* lysate-derived soluble fraction to accelerate wound closure process *in vitro*. Results, expressed as mean ± SEM of three experiments in duplicate, are relative to the % re-epithelialization at 20 h vs. relative T0 from monolayer scratching in the presence or absence of bacterial samples at 50 µg/mL (**Figure 14A**). Representative images from microscopic observations are also shown (**Figure 14B**). As expected, in accordance with our previous findings [39], the pre-treatment with AG strongly affected the physiological repair of control monolayer and significantly prevented the stimulating effect of all these bacterial samples in terms of monolayer repair rate expressed as % re-epithelialization vs. relative T0.

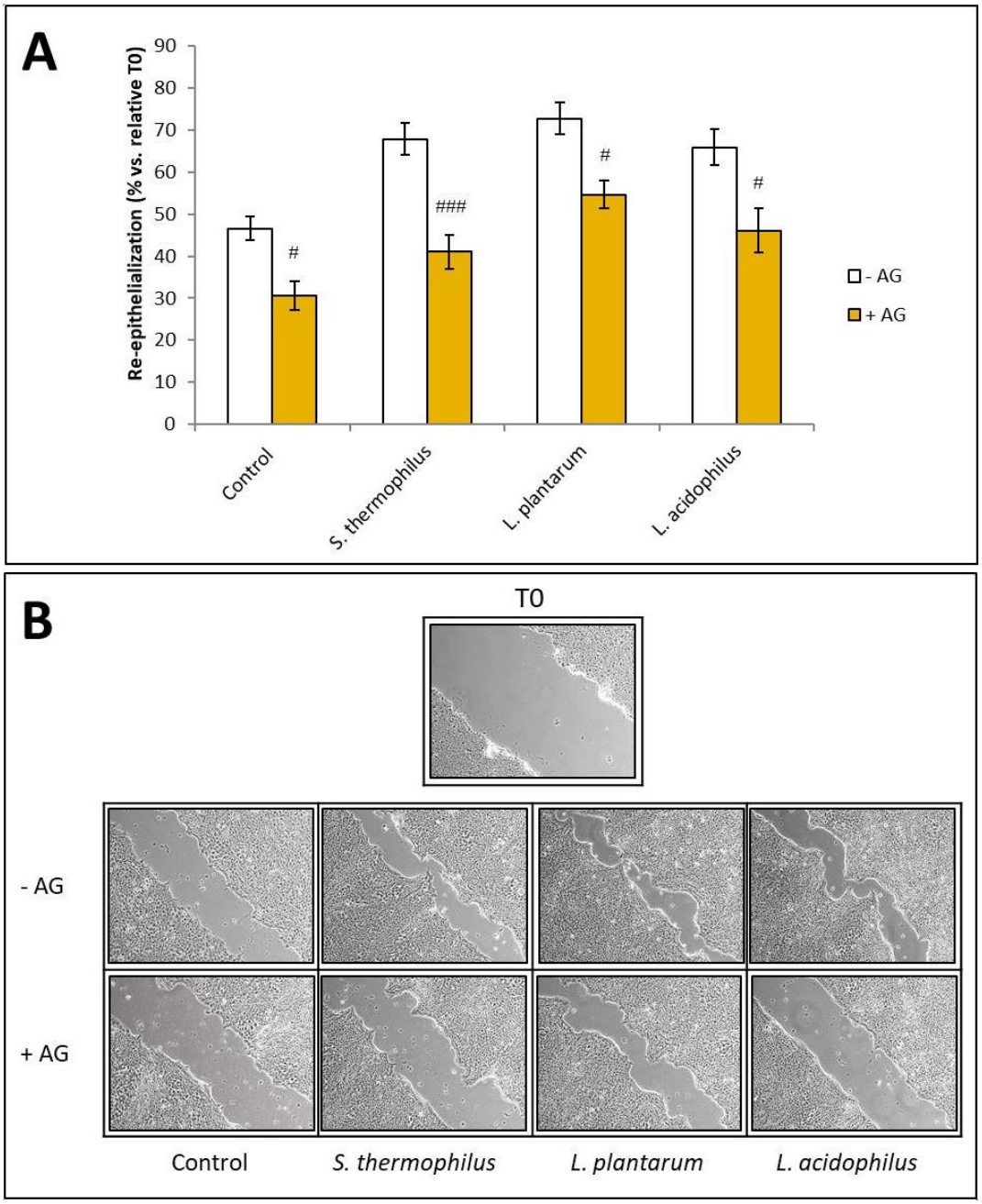


Figure 14. Effect of NOS2 inhibitor AG on the re-epithelialization of HaCaT scratched monolayers induced by *S. thermophilus, L. plantarum, and L. acidophilus*. (**A**) Effect of 15 min pre-treatment with 20 µM AG on the relative wound closure rate (%) of scratched monolayers with or without the indicated probiotic lysate-soluble fraction at 50 µg/mL for 20 h. Data are expressed as mean ± SEM of three independent experiments in duplicate. For comparative analysis of groups of data, a two-way analysis of variance (ANOVA) with *post hoc* Bonferroni test was used. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. each relative culture without AG. (**B**) Representative images of HaCaT cell line scratched monolayers pre-treated with or without 20 µM AG for 15 min and then with *S. thermophilus, L. plantarum* or *L. acidophilus* samples at 50 µg/mL for 20 h (10× magnification).

To verify the statistical relationship between the percentages of wound closure, NOS2 expression, and nitrite levels, Pearson's correlation test was used. Statistical analysis revealed a positive, strong, statistically significant correlation between NOS2

protein levels (fold vs. control) and wound closure (% re-epithelialization vs. relative T0) (r = 0.885*; P* < 0.0001) (**Figure 15A**). Similarly, Pearson's test revealed a strong correlation also between nitrite levels (fold vs. relative T0) and wound closure (% re-epithelialization vs. relative T0) (r = 0.908*; P* < 0.0001) (**Figure 15B**). Takentogether, these results, confirming the involvement of NO in the wound repair process, strongly suggest that the up-modulation of NOS2 expression and activity plays a crucial role in the re-epithelialization process elicited by the soluble fraction from probiotic lysates.

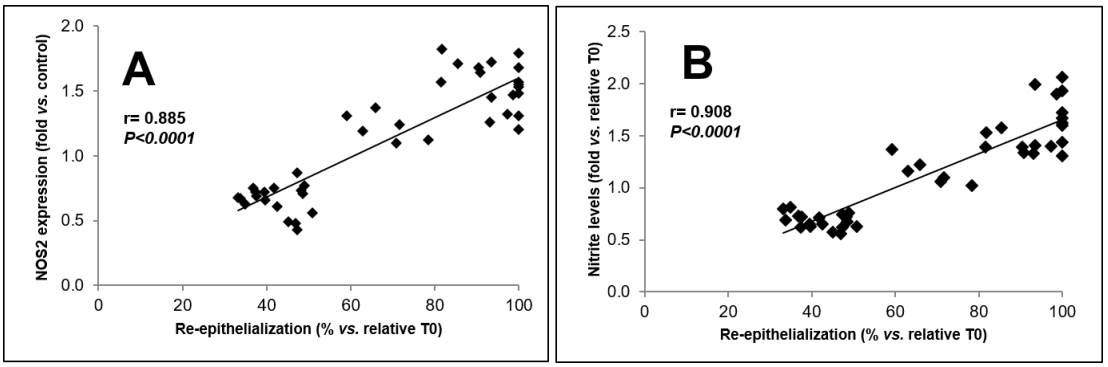


Figure 15. Analysis of the correlation between NOS2 expression, nitrite levels, and wound closure rate. (A) Pearson's test analysis of the correlation between NOS2 expression (fold vs. control) and wound closure rate expressed as % re-epithelialization vs. relative T0. **(B)** Pearson's test analysis of correlation between nitrite levels (fold vs. relative T0) and wound closure rate expressed as % re-epithelialization vs. relative T0.

5. RESULTS ON MODEL #2: INTESTINAL EPITHELIAL BARRIER IN VITRO

5.5 EFFECT OF VIVOMIXX TREATMENT ON VIABILITY AND PARACELLULAR PERMEABILITY OF AN IN VITRO INTESTINAL EPITHELIAL BARRIER MODEL.

No significant influence on cell count and viability was observed after treatment with Vivomixx formulation (not shown).

In order to evaluate the ability of Vivomixx to influence the epithelial integrity, TEER measurements were registered at different time intervals (0, 1, 3, and 6 h) on CaCo-2 untreated or treated with the probiotic mixture at 10^8 CFU/ml (final concentration). Results reported in **Figure 16A** show the TEER values expressed as percentage change from baseline and mean ± SEM of two independent experiments performed in duplicate. The Vivomixx addition, despite a slight increasing trend, did not affect significantly the values of TEER compared to control at all time intervals. In **Figure 16B** representative microscopy images from transwell chambers at 20X magnification with CaCo-2 monolayers untreated or treated with Vivomixx at 10^8 CFU/ml for 3 h are shown. In accordance with TEER results, the microscopy images showed no appearance of cell-free areas (holes) in the monolayer treated with Vivomixx mixture, thus suggesting that Vivomixx did not induce damage to the epithelial barrier, preserving the continuity of the monolayer surface.

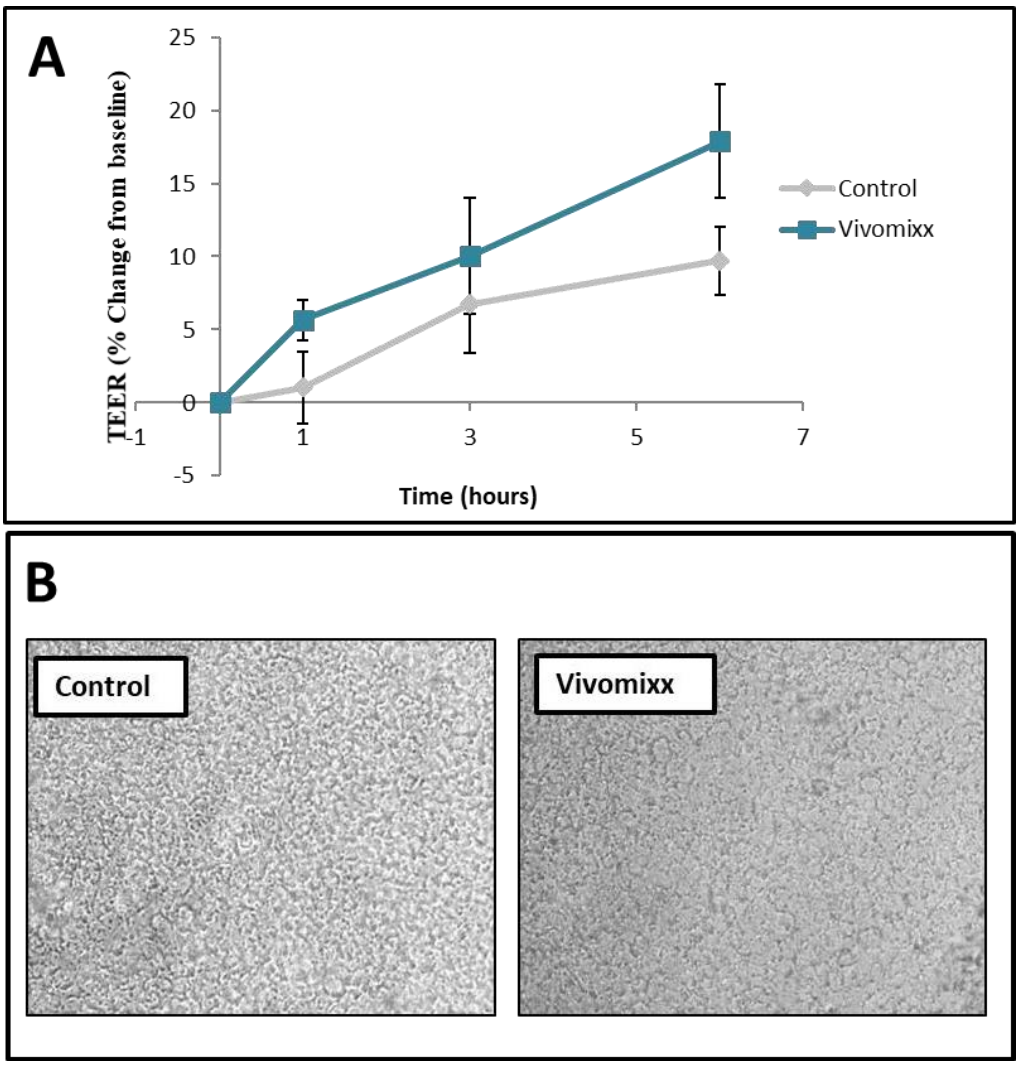


Figure 16. Effects Vivomixx on TEER levels. (A) TEER values expressed as % change of baseline over time (0-6 hours) in the presence or absence of Vivomixx at 10^8 CFU/ml. Data are expressed as mean ± SEM of two independent experiments performed in duplicate. Differences among mean values were assessed by two-way repeated measures ANOVA following by Bonferroni post-hoc test ($P > 0.05$). **(B)** Representative microscopy images from transwell chambers at 20X magnification with CaCo-2 monolayers untreated (control) or treated with Vivomixx at 10^8 CFU/ml for 3 h.

To evaluate the ability of Vivomixx formulation to influence paracellular permeability of differentiated CaCo-2 monolayers, the measurement of fluoresceinated dextran-4 amount through the monolayers was used. Flux of FITC-dextran-4 (FD4) in differentiated CaCo-2 cell monolayers treated or not with Vivomixx formulation for 3 hours was assayed as described in Materials and Methods' section. In agreement with the TEER values registered at 3 h treatment and reported in Figure 5, the exposure to Vivomixx formulation did not affect the FD4 flux when compared to untreated monolayer. The results expressed as μg/ml FITC-dextran recovered from basolateral

compartments are shown in **Figure 17** as means ± SEM of two independent experiments performed in duplicate.

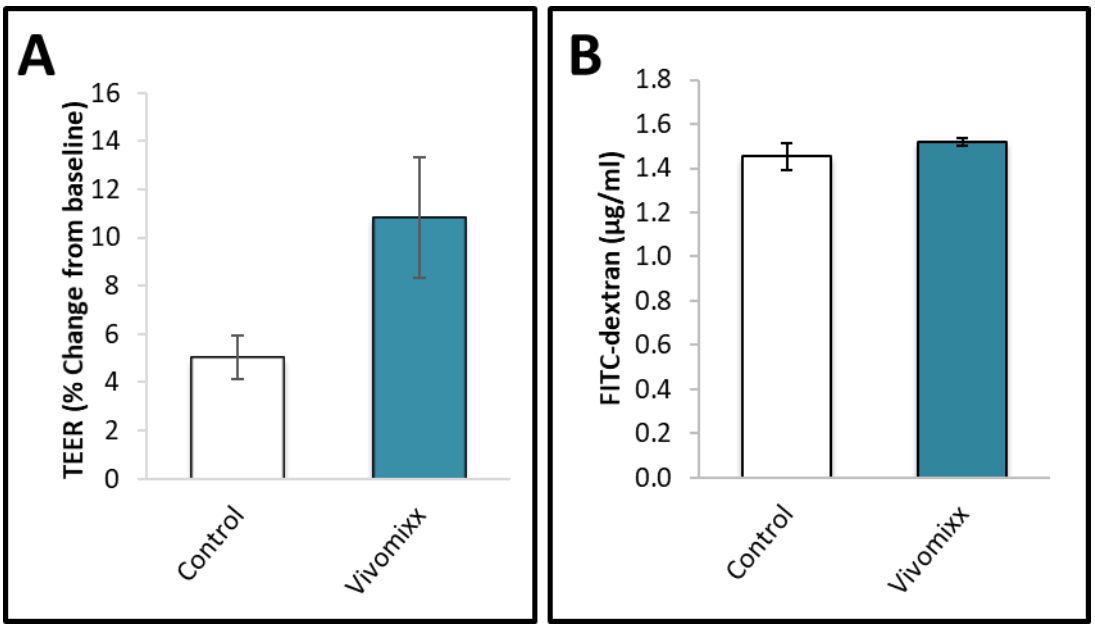


Figure 17. Effects of Vivomixx on TEER levels and FD4 flux. (A) TEER values expressed as % change of baseline at 3 hours in the presence or absence of Vivomixx at 10^8 CFU/ml. Data are expressed as mean ± SEM of three independent experiments performed in duplicate. Differences among mean values were assessed using a two-tailed t-test ($P > 0.05$ vs. Control). **(B)** FD4 flux across the differentiated CaCo-2 monolayers was measured after 3 h treatment in the presence or absence of Vivomixx at 10^8 CFU/ml. Results are expressed as mean ± SEM of two independent experiments performed in duplicate. Differences among mean values were assessed using a two-tailed t-test ($P > 0.05$ vs. Control).

5.6 EFFECTS OF VIVOMIXX ON ZO-1 AND OCCLUDIN LEVELS.

In order to further investigate the ability of Vivomixx preparation to influence the levels of TJ proteins, ZO-1 and occludin analysis by western blotting were performed in CaCo-2 cells before and after 3 h treatment with the bacterial samples. As shown in **Figure 18A**, treatment with Vivomixx preparations at 10^8 CFU/ml did not significantly alter the ZO-1 protein expression levels. On the other hand, the treated cells showed a significant increase of occludin level respect to control ($P < 0.001$). The results are expressed as mean ± SEM of three independent experiments in duplicate (ZO-1 or occludin expression, fold versus control). In **Figure 18B**, images from one representative experiment for ZO-1 and occluding levels, as analyzed by western blotting are shown.

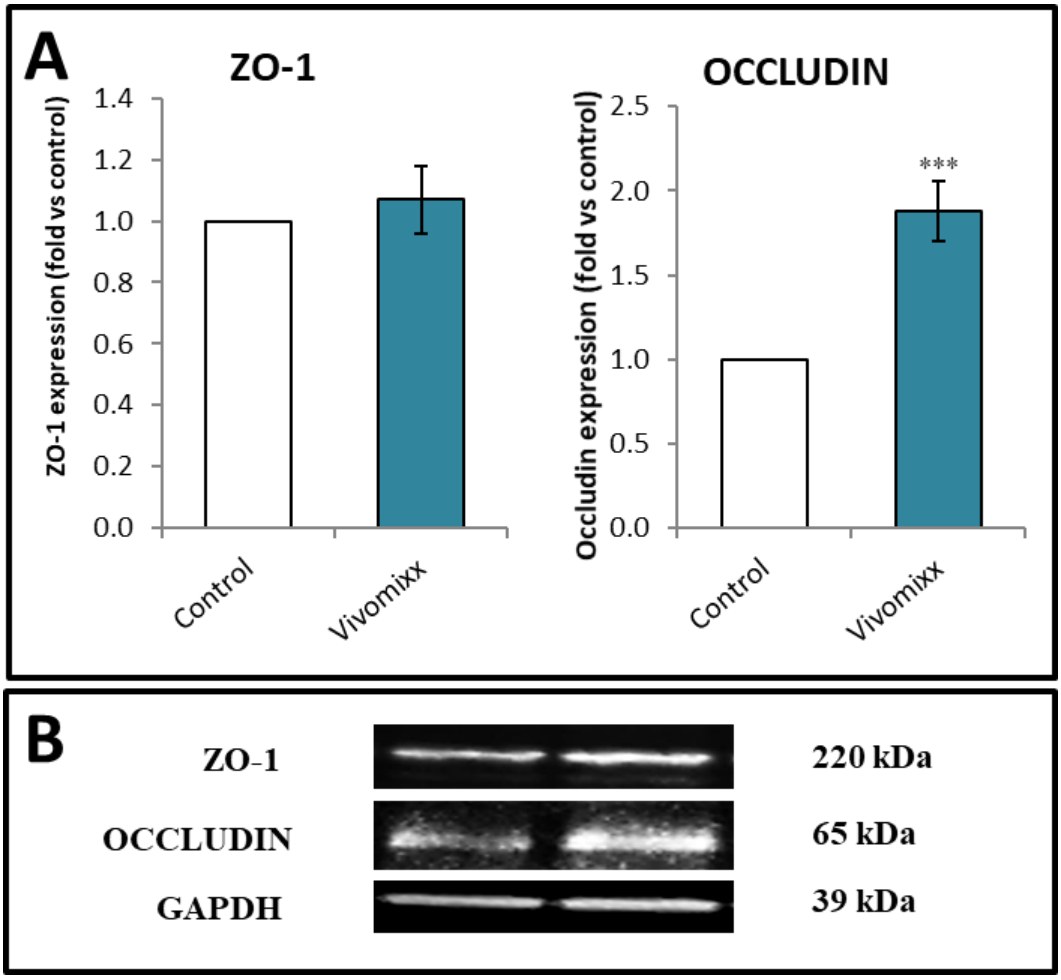


Figure 18. Effects of Vivomixx on TJ protein levels. (A) Densitometric analysis of western blot bands respectively for ZO-1 and occludin levels in CaCo-2 monolayers incubated for 3 h in the presence or absence of Vivomixx at 10^8 CFU/ml. Values were normalized to GAPDH, and the results are presented as fold increase vs. control and represent mean ± SEM of three independent experiments in duplicate. Differences among mean values were assessed using a two-tailed t-test (*** $P < 0.001$ vs. control). (B) Western blot images from one representative out of three independent experiments for ZO-1 and occludin protein levels are shown.

5.7 EFFECT OF VIVOMIXX ON PARACELLULAR PERMEABILITY IN HEAT-STRESSED CACO-2 DIFFERENTIATED MONOLAYERS.

In order to evaluate the effect of Vivomixx preparation on heat damage induced in CaCo-2 cells, experiments on TEER measurements and paracellular dextran flux after exposure of cells to 40°C for 10 minutes have been performed. To this aim, cell monolayers were incubated with Vivomixx at 10^8 CFU/ml for 4 h before heat treatment. Results shown in **Figure 19A** are expressed as mean ± SEM of two independent experiments in duplicate. Heat stress induced in control monolayers a drop in TEER values with ~20-30% reduction when compared to relative baseline values. Of note, pre-

treatment with Vivomixx totally prevented the TEER heat-induced fall, even inducing a slight increase in TEER value ($P < 0.05$ vs. heat-exposed control). In accordance, heat stress-induced FD4 flux increase could be totally prevented by pre-treatment with Vivomixx (**Figure 19B**) ($P < 0.01$ vs. heat-exposed control). Results are expressed as mean ± SEM of three independent experiments in duplicate.

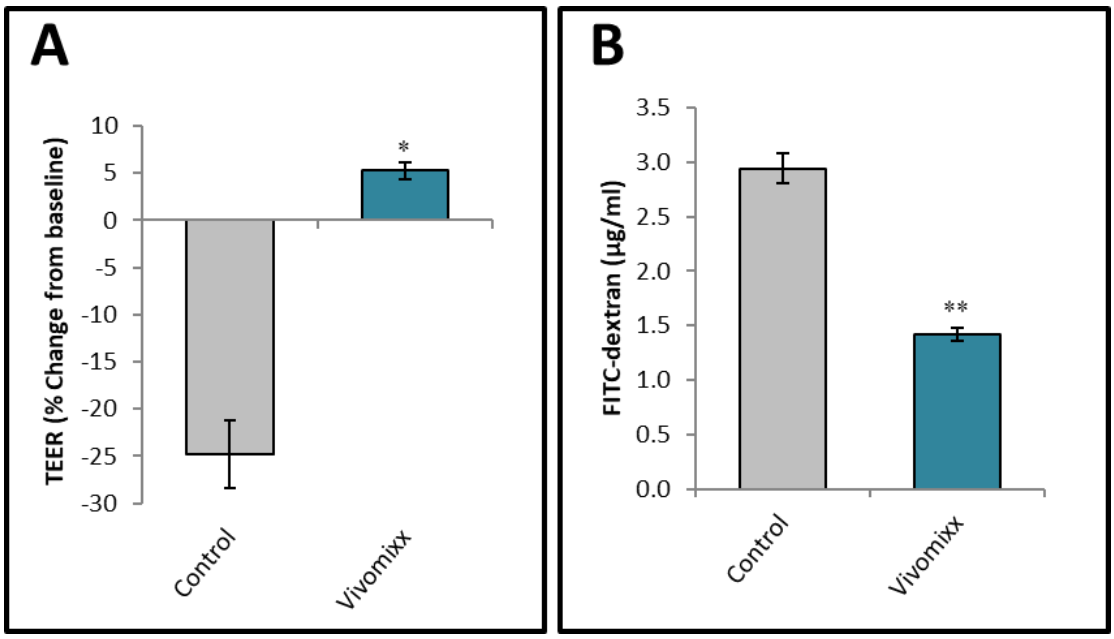


Figure 19. Effects of pre-treatment with Vivomixx on TEER values and FD4 flux in heat-stressed CaCo-2 differentiated monolayers. The effects of a 4 h pre-treatment with Vivomixx at 10^8 CFU/ml before heat stress at 40°C for 10 min are shown. (**A**) TEER values expressed as % change of baseline at 3 h after exposure of CaCo-2 monolayers to 40°C for 10 minutes. Data are presented as means ± SEM of two experiments performed in duplicate. Differences among mean values were assessed using a two-tailed t-test (* $P < 0.05$ vs. control). (**B**) FD4 flux data are expressed as means ± SEM of three experiments performed in duplicate. Differences among mean values were assessed using a two-tailed t-test (** $P < 0.01$ vs. control).

5.8 EFFECT OF VIVOMIXX ON ZO-1 AND OCCLUDIN LEVELS IN CACO-2 CELLS EXPOSED TO HEAT-INDUCED STRESS.

The influence of pre-treatment with Vivomixx mixture on the expression of ZO-1/occludin in CaCo-2 cells exposed to heat-induced stress was also investigated. Results are shown in **Figures 20** and expressed as mean ± SEM of three independent experiments in duplicate. Representative western blot images are also reported. Heating of cells for 10 minutes at 40°C induced a significant reduction of ZO-1 level ($P < 0.05$ vs. non-exposed untreated, **Figure 20A**). No significant change was instead observed in occludin level between unexposed controls and cell monolayers exposed to heating for a short period. Pre-treatment with Vivomixx for 4 hours at doses of 10^8 CFU/ml fully

preserved ZO-1 expression levels, which were similar to those detected in untreated unexposed cells and significantly higher when compared to heat-stressed control ($P < 0.01$). In accordance with the results reported in Figure 19, also in these conditions, Vivomixx was able to strongly elevate occludin levels, which resulted significantly higher either versus unexposed or heat-exposed controls ($P < 0.05$ vs. both controls, **Figure 20B**).

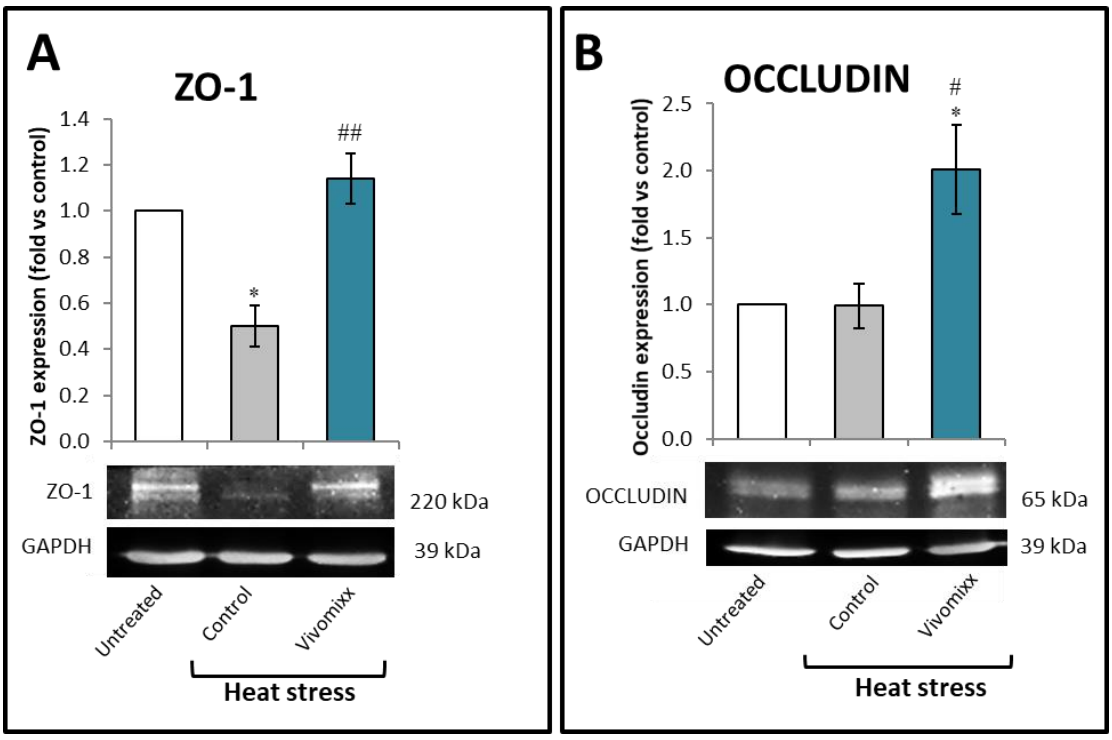


Figure 20. Effects of pre-treatment with Vivomixx on TJ protein levels in heat-stressed CaCo-2 differentiated monolayers. The effects of a 4 h pre-treatment with Vivomixx at 10^8 CFU/ml before heat stress at 40°C for 10 min are shown. (**A**) and (**B**) Densitometry of bands from western blot analysis respectively of ZO-1 and occludin in CaCo-2 cells. Values were normalized to GAPDH. Histogram bars are relative to fold vs. untreated cells. Results are expressed as mean values ± SEM of three independent experiments. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (* $P < 0.05$ vs. untreated; # $P < 0.05$ and ## $P < 0.01$ vs. heat-exposed control). Images from one representative out of three independent experiments for ZO-1 and occludin protein levels analyzed by western blotting are shown.

6. DISCUSSION

In the first part of the present study, the effect of the soluble fraction from lysates of seven different probiotic strains on the re-epithelialization process in an *in vitro wound healing model* was studied. Considering the crucial barrier function of the epidermis, when an injury occurs, it is necessary to re-establish tissue integrity as fast and efficiently as possible, through the re-epithelialization process. Keratinocyte proliferation and migration represent an essential step in the re-epithelialization process during wound healing [304]. There is a growing body of research involving the use of a topical application of probiotics in dermatology with benefits in atopic dermatitis, acne, seborrheic dermatitis, and wound healing [16,305,306]. The use of probiotic formulations would thus represent a valid alternative approach to overcome the existing problems of actual wound therapy approaches, including the high costs, the long manufacturing times, and the increase in antibiotic resistance. The results of a recent meta-analysis from controlled *in vivo* studies testing the efficacy of probiotics for skin lesions in animal models strongly support that topical probiotic administration is an effective pharmacological treatment for cutaneous wounds [307]. However, further studies are needed both to identify probiotics or any combinations of them in terms of therapeutic efficacy and to fully define the underlying mechanisms. With this purpose, the ability of different probiotic samples to influence the re-epithelialization process *in vitro* was compared. The soluble fraction from *S. thermophilus, L. plantarum*, and *L. acidophilus*, lysates promoted the re-epithelialization of scratched HaCat monolayers. In contrast *B. longum, B. infantis*, and *B. breve* inhibited the monolayer repair process, whereas *L. bulgaricus* had no effect. Given the key role of the NO generation in the wound healing process and with the aim to identify the mechanisms mediating the observed effects of the probiotic samples, the possible involvement of the NOS2/NO system in the ability of probiotics to influence wound healing *in vitro* was examined. The acceleration of re-epithelialization induced by *S. thermophilus, L. plantarum*, and *L. acidophilus*, was associated with an increase of NOS2 expression and activity, as demonstrated by immunoblotting data and nitrite level assay. Of note, the ability of the soluble fraction from *S. thermophilus, L. plantarum*, and *L. acidophilus* lysates to accelerate re-epithelialization was abrogated by the inhibition of NOS2 by AG, thus

confirming that the beneficial effect of these probiotics may be mediated by NOS2/NO pathway. On the other hand, *B. infantis*, *B. breve*, and *B. longum*, significantly downmodulated NOS2 expression and activity in scratched HaCat monolayers. *L. bulgaricus*, also in this context, did show no effect. **Figure 21**, with the aid of arrows, summarizes the effects of all used probiotics on keratinocyte re-epithelialization, NOS2 expression, and nitrite levels. Taken together, these results strongly suggest that the pro- or anti-healing properties of probiotics used are strictly strain-dependent and associated with their ability to up- or down-modulate NOS2 expression and activity, as also supported by the Pearson's correlation analyses.

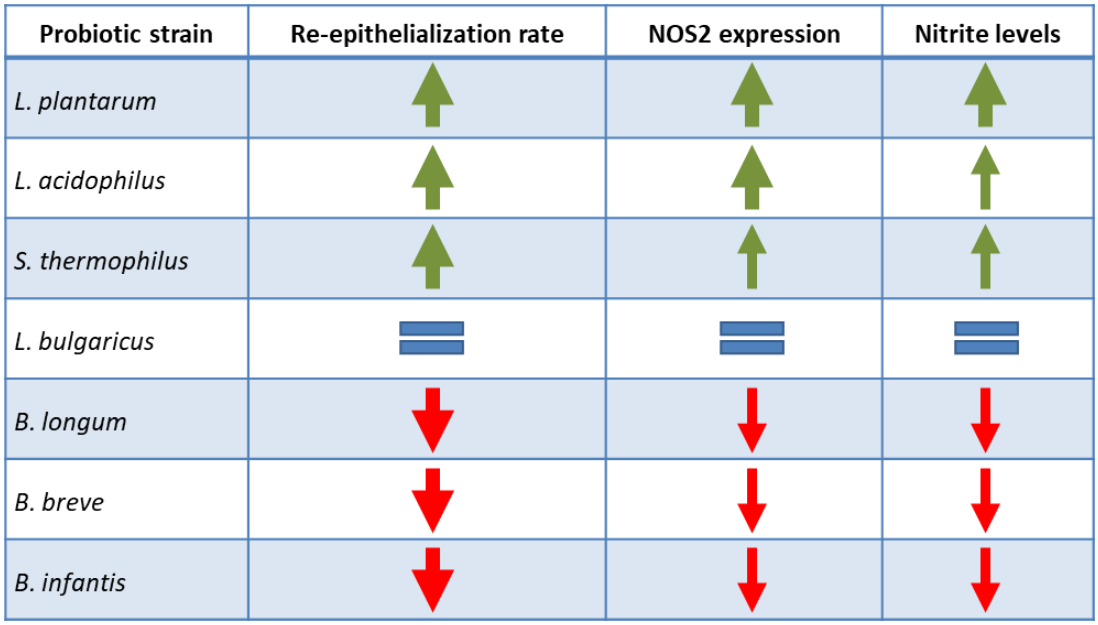


Figure 21. Effects of the soluble fraction from lysates of the used probiotic strains on scratched HaCat monolayers: Overview of the re-epithelialization potential of the used probiotic strains as well as their ability to influence NOS2 protein expression and activity.

These data extend the spectrum of the mechanisms underlying the effects of specific probiotics on the re-epithelialization process and may further justify their use in the topical treatment of skin wounds. Even if further *in vivo* studies should be conducted to confirm their potential therapeutic use, it will be of interest to firstly verify the reproducibility of the observed effects on HaCat cell line, also on primary keratinocytes, as well as other cell types involved in skin wound healing process (i.e., fibroblasts, endothelial cells), focusing on the biomolecular mechanism behind the observed effects. In addition, these findings strongly support that the choice of the probiotic strains should

also be considered of crucial importance, as the effects of these bacteria are highly strain-specific.

In the second part of this work, the effect of Vivomixx, a multistrain probiotic formulation commercially available, was analyzed on an *in vitro* intestinal epithelial barrier model. The maintenance or enhancement of intestinal barrier function is a beneficial property exerted by some probiotics and/or relative secreted bioactive factors [297-300]. Of note, the original VSL#3, now named Vivomixx, has been recently reported to reduce colonic inflammation and improve intestinal barrier function in Muc2 mucin-deficient mice [308].

Both active and passive transport can be investigated *in vitro* using the Caco-2 line, derived from a human colorectal adenocarcinoma, and able to spontaneously differentiate into a polarized monolayer when grown on porous inserts. In fact, the Caco-2 cells cultured on a semipermeable filter insert which defines a partition for upper and lower compartments, close up and tighten becoming similar to the intestinal barrier composed by enterocytes with microvilli (brush border) on the apical side and a basolateral side. They also express TJ proteins, similar to the small intestine], that restrict the diffusion of substances across the barrier [309].

For these reasons, in this study, the effect of Vivomixx on human colon cancer cells (CaCo-2) have been evaluated. Vivomixx did not affect TEER values and epithelial permeability of polarized monolayers assessed by measuring the FITC-labeled dextran flux from the apical to basolateral chambers. Interestingly, it could totally prevent the heat-induced loss epithelial barrier integrity and was also able to maintain the basal dextran flux. Even if the mechanisms are not fully understood, several studies have been focused on probiotic ability to prevent alterations to barrier integrity and tight junctions (TJ) using *in vivo* and *in vitro* models [95,297,300,301,309]. TJ represents a very dynamic structure consisting of transmembrane proteins located at the apical contact point between cells in epithelial and endothelial tissue which form a continuous barrier between epithelial cells and other tissues, regulating a selective paracellular transport across the epithelium, and preserving the integrity of the epithelial barrier [93,310-315]. In accordance, VSL#3 (De Simone formulation), has been shown to enhance TJ integrity [316-318], reduce dextran sodium sulphate-induced colitis in rodent models by

preventing TJ alterations [308,319,320] and improve human intestinal health [321-323]. In our hands, Vivomixx did not affect ZO-1 expression and induced an increase of occludin levels. Of note, it increased occludin expression also in the presence of heat stress, demonstrating to have a protective effect against intestinal injury.

Our findings confirm that CaCo-2 cells represent an optimum *in vitro* model for studies on intestinal barrier functions due to their ability to spontaneously differentiate into a polarized monolayer and to express tight junction proteins, similar to the small intestine. The intestinal mucosa consists of an epithelial layer, the lamina propria and the muscularis mucosa where the epithelial layer is the guardian hurdle for nutrient absorption, drug permeation and toxic substances exclusion. So, humans rely on an efficient intestinal barrier to protect themselves against pathogens and pathobionts while hosting beneficial bacteria.

Our data support that the probiotic formulations can be analyzed on transepithelial electrical resistance, dextran flux and expression TJ proteins i.e. zonulin-1 (ZO-1) and occludin, in the absence or presence of a heat stress-related damage of cell monolayer, to detect *in vitro* functional differences which could be clinically relevant. Indeed, the bioavailability of nutrients, drugs or other substances administered orally depends on intestinal barrier integrity that can be compromised in various diseases such as inflammatory bowel diseases, infectious enteritis, necrotizing enterocolitis, serious liver diseases.

Variability in probiotic manufacturing may affect their properties, with potential implications for their efficacy and safety. Genetically identical bacterial strains, if produced differently, could not maintain the same probiotic properties. For example, significant changes in the *in vitro* properties of early *L. rhamnosus GG* strains were reported, when functional tests were done on fifteen different *Lactobacillus rhamnosus GG* isolates with 100% sequence homology [324]. This fact, attributed to different production processes and a long-term series of re-inoculations, has also been described for *L. acidophilus* to such an extent that the outcome of the human intervention studies may be different [312]. Recently, the influence of technological treatments on the functionality of *Bifidobacterium lactis INL1* has been confirmed [313] and the antifungal activity of *Lactobacillus pentosus ŁOCK 0979* has been shown to be dependent from the presence of polyols and galactosyl-polyols in the culture medium

[325]. Therefore, it is evident that genomic characterization of probiotic strains is not an appropriate solution to establish the consistency of a probiotic production. Functional studies are needed to assess if production changes have a substantive impact on probiotic efficacy and safety.

Each step and variable involved in the culture production. From growth conditions, substrates, and protectants to food formulation, processing, and storage conditions, may affect probiotic properties [317,318]. Recently, the group of Prof. Cifone reported that the VSL#3 formulations manufactured in the USA and Italy have a different effect on tumor cell lines and wound healing [293,294]. The same discrepancies between VSL#3 formulations manufactured at different sites have been reported either by Biagioli et al. in animal models of IBDs [295] or by Trinchieri et al. in HIV-infected subjects [296]. These observations may have a major impact on patient safety and on the liability of doctors when they prescribe a probiotic formulation made with different processes at different production sites, without properly informing the patients.

Once human intervention studies have documented that microorganisms qualify as probiotics, the next step will need to be taken to see if the strains can be cultured at an industrial scale and if they can be successfully incorporated in consumer products. Preferably, this part of probiotic commercialization will run in parallel with clinical trials, to avoid studying a non-commercializable strain. Culturing at a large scale and industrial processing set very different requirements for strains than laboratory scale culturing; also, medium requirements are very different due to cost and other factors.

As correctly stated by Fenster et al in a recent review [326], "*For manufacturing, an intricate production process is required that ensures both high yield and stability and must also be able to meet requirements such as the absence of specific allergens, which precludes some obvious culture media ingredients* **(Figure 22)**. *Reproducibility is important to ensure constant high performance and quality. To ensure this, quality control throughout the whole production process, from raw materials to the final product, is essential, as is the documentation of this quality control*". The same Authors pointed out that *"to ensure the consistent high quality of the strains, a quality control program needs to be established to assure the consistent quality of everything, from ingredients to final product, and a quality assurance program needs to be in place to*

run reliable production processes. This all requires rigorous documentation of procedures and results. Once high-quality probiotic bulk has been produced, the strain(s) need to be incorporated into consumer products. These products have different requirements, from shelf life to storage conditions and product composition. In any case, a minimal efficacious dose should be delivered to the consumer at the end of shelf life. Probiotics, being live microorganisms, make this all challenging. However, by choosing the right strains, culture conditions, and product manufacturing, much can be achieved, and the investigated health benefits can be delivered to the consumer".

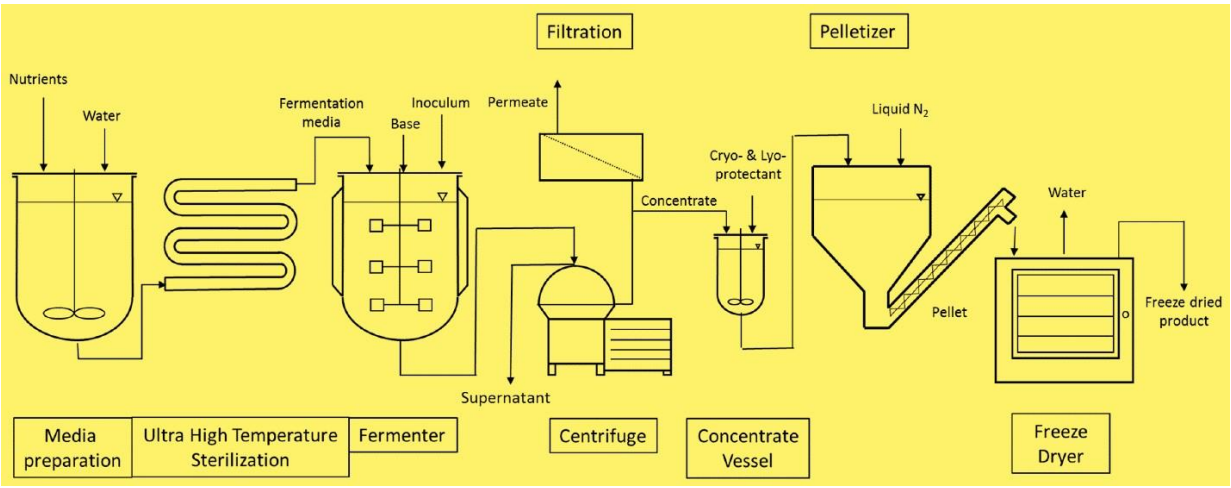


Figure 22. Schematic representation of the production of probiotics for dietary supplements and dairy starter culture strains.

In conclusion, a careful selection of the probiotic agent, standardization of the dose and detailed characterization of the beneficial effects are essential when considering use of a probiotic for the dietary or topical management of serious diseases. However, changes in the manufacturing processes, equipment or facilities can result in differences in the product itself due to the live nature of probiotics. The need to reconfirm safety and/or efficacy for any probiotic product made at a different factory is therefore mandatory.

In vitro models, such as those used for this work can be very useful and suitable for an *in vitro* verification of the effects of a probiotic or a probiotic combination.

7. REFERENCES

1. Food and Agriculture Organization of the United Nations/World Health Organization FAO/WHO. Guidelines for the Evaluation of Probiotics in Food; Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food: London, ON, Canada, 2002.

2. Leeming ER, Johnson AJ, Spector TD, and Le Roy C. Effect of diet on the gut microbiota: Rethinking intervention duration. Nutrients. 2019; 11(12):2862.

3. Kamada N, Kim YG, Sham HP, Vallance BA, Puente JL, Martens EC, Núñez G. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. Science. 2012; 336(6086):1325–1329.

4. Hernández-Chirlaque C, Aranda CJ, Ocón B, Capitán-Cañadas F, Ortega-González M, Carrero JJ, Suárez MD, Zarzuelo A, Sánchez de Medina F, Martínez-Augustin O. Germ-free and antibiotic-treated mice are highly susceptible to epithelial injury in DSS colitis. J. Crohns Colitis. 2016; 10(11):1324–1335.

5. Breton J, Daniel C, Dewulf J, Pothion S, Froux N, Sauty M, Thomas P, Pot B, Foligné B. Gut microbiota limits heavy metals burden caused by chronic oral exposure. Toxicol. Lett. 2013; 222(2):132–138.

6. Luczynski P, McVey Neufeld KA, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a bubble: Using germ-free animals to assess the influence of the gut microbiota on brain and behavior. Int. J. Neuropsychopharmacol. 2016; 19(8)pii:pyw020.

7. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. Clin. Exp. Allergy. 2014; 44(6):842–850.

8. Thushara RM, Gangadaran S, Solati Z, Moghadasian MH. Cardiovascular benefits of probiotics: A review of experimental and clinical studies. Food Funct. 2016; 7(2):632–642.

9. Aggarwal J, Swami G, Kumar M. Probiotics and their effects on metabolic diseases: An update. J. Clin. Diagn. Res. 2013; 7(1):173–177.

10. Collins FL, Rios-Arce ND, Schepper JD, Parameswaran N, McCabe LR. The potential of probiotics as a therapy for osteoporosis. Microbiol. Spectr. 2017; 5(4):1–26.

11. Roudsari MR, Karimi R, Sohrabvandi S, Mortazavian AM. Health effects of probiotics on the skin. Crit. Rev. Food. Sci. Nutr. 2015; 55(9):1219–1240.

12. Slyepchenko A, Carvalho AF, Cha DS, Kasper S, McIntyre RS. Gut emotions-mechanisms of action of probiotics as novel therapeutic targets for depression and anxiety disorders. CNS Neurol. Disord. Drug Targets. 2014; 13(10):1770–1786.

13. Westfall S, Lomis N, Kahouli I, Dia SY, Singh SP, Prakash S. Microbiome, probiotics and neurodegenerative diseases: Deciphering the gut brain axis. Cell Mol. Life Sci. 2017; 74(20):3769–3787.

14. Islam SU. Clinical uses of probiotics. Medicine. 2016; 95(5):e2658.

15. George Kerry R, Patra JK, Gouda S, Park Y, Shin HS, Das G. Benefaction of probiotics for human health: A review. J. Food Drug Anal. 2018; 26(3):927–939.

16. Kiousi DE, Karapetsas A, Karolidou K, Panayiotidis MI, Pappa A, Galanis A. Probiotics in extraintestinal diseases: Current trends and new directions. Nutrients. 2019; 11(4):e788.

17. Homayouni-Rad A, Soroush AR, Khalili L, Norouzi-Panahi L, Kasaie Z, Ejtahed HS. Diabetes management by probiotics: current knowledge and future pespective. Int. J. Vitam. Nutr. Res. 2016; 86(3-4):215-227.

18. Hojsak I, Snovak N, Abdović S, Szajewska H, Misak Z, Kolacek S. *Lactobacillus* GG in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers: a randomized, double-blind, placebo-controlled trial. Clin. Nutr. 2010; 29(3):312–316.

19. Demers M, Dagnault A, Desjardins J. A randomized double-blind controlled trial: impact of probiotics on diarrhea in patients treated with pelvic radiation. Clin. Nutr. 2014; 33(5):761–767.

20. Ringel-Kulka T, Palsson OS, Maier D, Carroll I, Galanko JA, Leyer G, Ringel Y. Probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders: A double-blind study. J. Clin. Gastroenterol. 2011; 45(6):518–525.

21. Reid G, Bruce AW, Fraser N, Heinemann C, Owen J, Henning B. Oral probiotics can resolve urogenital infections. FEMS Immunol. Med. Microbiol. 2001; 30(1):49–52.

22. Ganji-Arjenaki M, Rafieian-Kopaei M. Probiotics are a good choice in remission of inflammatory bowel diseases: A meta analysis and systematic review. J. Cell Physiol. 2018; 233(3):2091-2103

23. Tsilingiri K, Barbosa T, Penna G, Caprioli F, Sonzogni A, Viale G, Rescigno M. Probiotic and postbiotic activity in health and disease: Comparison on a novel polarised *ex-vivo* organ culture model. Gut. 2012; 61(7):1007–1015.

24. Volz T, Skabytska Y, Guenova E, Chen KM, Frick JS, Kirschning CJ, Kaesler S, Röcken M, Biedermann T. Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells. J. Invest. Dermatol. 2014; 134(1):96–104.

25. Christensen GJ, Brüggemann H. Bacterial skin commensals and their role as host guardians. Benef. Microbes. 2014; 5(2):201–215.

26. Yousefi B, Eslami M, Ghasemian A, Kokhaei P, Salek Farrokhi A, Darabi N. Probiotics importance and their immunomodulatory properties. J. Cell. Physiol. 2019; 234(6):8008-8018.

27. Plaza-Diaz J, Ruiz-Ojeda FJ, Gil-Campos M, Gil A. Mechanisms of Action of Probiotics. Adv. Nutr. 2019; 10(S1):S49-S66.

28. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 2014; 11(8):506–514.

29. Lee YK. Selection and maintenance of probiotic microorganisms. Handbook of Probiotics and Prebiotics. In: Lee YK, Salminen S, editors. Wiley-VCH; Weinheim, Germany. 2009; pp. 177–187.

30. Sanders ME, Lenoir-Wijnkoop I, Salminen S, Merenstein DJ, Gibson GR, Petschow BW, Nieuwdorp M, Tancredi DJ, Cifelli CJ, Jacques P, Pot B. Probiotics and prebiotics: Prospects for public health and nutritional recommendations. Ann. N. Y. Acad. Sci. 2014; 1309:19–29.

31. Fijan S. Microorganisms with claimed probiotic properties: An overview of recent literature. Int. J. Environ. Res. Public Health 2014; 11(5):4745–4767.

32. Anadón A, Martínez-Larrańaga MR, Martínez MA. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol. 2006; 45(1):91–95.

33. Gaggia F, Mattarelli P, Biavati B. Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol. 2010; 141(S1):S15–S28.

34. Sanders ME, Merenstein DJ, Reid G. Gibson GR, Rastall RA. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. Nat. Rev. Gastroenterol. Hepatol. 2019; 16(10):605-616.

35. Oelschlaeger TA. Mechanisms of probiotic actions—A review. Int. J. Med. Microbiol. 2010; 300(1):57–62.

36. Cremonini F, di Caro S, Nista EC, Bartolozzi F, Capelli G, Gasbarrini G, Gasbarrini A. Meta-analysis: The effect of probiotic administration on antibiotic associated diarrhoea. Aliment. Pharmacol. Ther. 2002; 16(8):1461–1467.

37. Johnston BC, Supina AL, Vohra S. Probiotics for pediatric antibiotic-associated diarrhea: A meta-analysis of randomized placebo-controlled trials. Can. Med. Assoc. J. 2006; 175(4):377–383.

38. Schoster A, Kokotovic B, Permin A, Pedersen PD, Dal Bello F, Guardabassi L. *In Vitro* inhibition of Clostridium difficile and Clostridium perfringens by commercial probiotic strains. Anaerobe. 2013; 20:36–41.

39. JimmySaint-Cyr M, Haddad N, Taminiau B, Poezevara T, Quesne S, Amelot M, Daube G, Chemaly M, Dousset X, Guyard-Nicodème M. Use of the potential probiotic strain *Lactobacillus salivarius* SMXD51 to control *Campylobacter jejuni* in broilers. Int. J. Food Microbiol. 2017; 247:9–17.

40. Carter A, Adams M, La Ragione RM, WoodWard MJ. Colonisation of poultry by *Salmonella Enteritidis* S1400 is reduced by combined administration of *Lactobacillus salivarius* 59 and *Enterococcus faecium* PXN-33. Vet. Microbiol. 2017; 199:100–107.

41. Chingwaru W, Vidmar J. Potential of Zimbabwean commercial probiotic products and strains of *Lactobacillus plantarum* as prophylaxis and therapy against diarrhoea caused by *Escherichia coli* in children. Asian Pac. J. Trop. Med. 2017; 10:57–63.

42. Hussain SA, Patil GR, Reddi S, Yadav V, Pothuraju R, Singh RRB, Kapila S. Aloe vera (Aloe barbadensis Miller) supplemented probiotic lassi prevents Shigella infiltration from epithelial barrier into systemic blood flow in mice model. Microb. Pathog. 2017; 102:143–147

43. Sikorska H, Smoragiewicz W. Role of probiotics in the prevention and treatment of ethicillin-resistant *Staphylococcus aureus* infections. Int. J. Antimicrob. Agents. 2013; 42(6):475–481.

44. De Montijo-Prieto S, Moreno E, Bergillos-Meca T, Lasserrot A, Ruiz-López M, Ruiz-Bravo A, Jimenez-Valera M. A *Lactobacillus plantarum* strain isolated from kefir protects against intestinal

infection with *Yersinia enterocolitica* O9 and modulates immunity in mice. Res. Microbiol. 2015; 166(8):626–632.

45. Thomas DW, Greer F. Probiotics and prebiotics in pediatrics. Pediatrics. 2010; 126(6):1217–1231.

46. Kumar S, Bansal A, Chakrabarti A, Singhi S. Evaluation of efficacy of probiotics in prevention of Candida colonization in a PICU—A randomized controlled trial. Crit. Care Med. 2013; 41(2):565–572.

47. Nase L, Hatakka K, Savilahti E. Effect of long-term consumption of *Lactobacillus* GG in milk on dental caries and caries risk in children. Caries Res. 2001; 35(6):412–420.

48. Della Riccia D, Bizzini F, Perilli MG, Polimeni A, Trinchieri V, Amicosante G, Cifone MG. Anti-inflammatory effects of *Lactobacillus brevis* (CD2) on periodontal disease. Oral Dis. 2007; 13(4):376-385.

49. Li P, Gu Q. Complete genome sequence of *Lactobacillus plantarum* LZ95, a potential probiotic strain producing bacteriocins and B-group vitamin riboflavin. J. Biotechnol. 2016; 229:1–2.

50. Gu Q, Zhang C, Song D, Li P, Zhu X. Enhancing vitamin B12 content in soy-yogurt by *Lactobacillus reuteri*. Int. J. Food Microbiol. 2015; 206:56–59.

51. Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M. Folate production by bifidobacteria as a potential probiotic property. Appl. Environ. Microbiol. 2007; 73(1):179–185.

52. Ishikawa H, Akedo I, Otani T, Suzuki T, Nakamura T, Takeyama I, Ishiguro S, Miyaoka E, Sobue T, Kakizoe T. Randomized trial of dietary fiber and *Lactobacillus casei* administration for prevention of colorectal tumors. Int. J. Cancer. 2005; 116(5):762–767.

53. Schellenberg J, Smoragiewicz W, Karska-Wysocki B. A rapid method combining immunofluorescence and flow cytometry for improved understanding of competitive interactions between lactic acid bacteria (LAB) and methicillin-resistant *S. aureus* (MRSA) in mixed culture. J. Microbiol. Methods. 2006; 65(1):1–9.

54. Markowiak P, Śliżewska K. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients. 2017; 9(9):E1021.

55. Monteagudo-Mera A, Rastall RA, Gibson GR, Charalampopoulos D, Chatzifragkou A. Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. Appl. Microbiol. Biotechnol. 2019; 103(16):6463-6472.

56. Begley M, Hill C, Gahan CGM. Bile salt hydrolase activity in probiotics. Appl. Environ. Microbiol. 2006; 72(3):1729–1738.

57. Weinberg ED. The *Lactobacillus* anomaly: Total iron abstinence. Perspect. Biol. Med. 1997; 40(4):578–583.

58. Elli M, Zink R, Rytz A, Reniero R, Morelli L. Iron requirement of *Lactobacillus* spp. in completely chemically defined growth media. J. Appl. Microbiol. 2000; 88(4):695–703.

59. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J. Gastroenterol. 2009; 44(1):26–46.

60. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic Mechanisms of Action. Ann. Nutr. Metab. 2012; 61(2):160-174.

61. Azad MAK, Sarker M, Wan D. Immunomodulatory Effects of Probiotics on Cytokine Profiles. Biomed. Res. Int. 2018; 2018:8063647.

62. Marteau P, Shanahan F. Basic aspects and pharmacology of probiotics: An overview of pharmacokinetics, mechanisms of action and side-effects. Best Pract. Res. Clin. Gastroenterol. 2003; 17(5):725–740.

63. Schatzmayr G, Zehner F, Taubel M, Schatzmayr D, Klimitsch A, Loibner AP, Binder EM. Microbiologicals for deactivating mycotoxins. Mol. Nutr. Food Res. 2006; 50(6):543–551.

64. Nikbakht Nasrabadi E, Jamaluddin R, Abdul Mutalib MS, Khaza'ai H, Khalesi S, Mohd Redzwan S. Reduction of aflatoxin level in aflatoxin-induced rats by the activity of probiotic *Lactobacillus casei* strain Shirota. J. Appl. Microbiol. 2013; 114(5):1507–1515.

65. McCormick SP. Microbial detoxification of mycotoxins. J. Chem. Ecol. 2013; 39(7):907–918.

66. Plaza-Díaz J, Ruiz-Ojeda FJ, Vilchez-Padial LM, Gil A. Evidence of the anti-inflammatory effects of probiotics and synbiotics in intestinal chronic diseases. Nutrients. 2017; 9(6):E555.

67. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012; 142(1):46–54.

68. Wedlake L, Slack N, Andreyev HJ, Whelan K. Fiber in the treatment and maintenance of inflammatory bowel disease: A systematic review of randomized controlled trials. Inflamm. Bowel Dis. 2014; 20(3):576–586.

69. Abraham C, Cho JH. Inflammatory bowel disease. N. Engl. J. Med. 2009; 361(21):2066–2078.

70. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu. Rev. Immunol. 2010; 28:573–621.

71. Derikx LA, Dieleman LA, Hoentjen F. Probiotics and prebiotics in ulcerative colitis. Best Pract. Res. Clin. Gastroenterol. 2016; 30(1):55–71.

72. Magro F, Gionchetti P, Eliakim R, Ardizzone S, Armuzzi A, Barreiro-de Acosta M, Burisch J, Gecse KB, Hart AL, Hindryckx P, Langner C, Limdi JK, Pellino G, Zagórowicz E, Raine T, Harbord M, Rieder F; European Crohn's and Colitis Organisation [ECCO]. Third European Evidence-Based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 1: Definitions, diagnosis, extra-intestinal manifestations, pregnancy, cancer surveillance, surgery, and ileo-anal pouch disorders. J. Crohns Colitis. 2017; 11(6):1–39.

73. O'Toole PW, Cooney JC. Probiotic bacteria influence the composition and function of the intestinal microbiota. Interdiscip. Perspect. Infect. Dis. 2008; 2008:175285.

74. Ringel Y, Carroll IM. Alterations in the intestinal microbiota and functional bowel symptoms. Gastrointest. Endosc. Clin. N. Am. 2009; 19(1):141–150.

75. Fontana L, Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gil A. Sources, isolation, characterisation and evaluation of probiotics. Br. J. Nutr. 2013; 109(S2):S35–S50.

76. Mann ER, You J, Horneffer-van der Sluis V, Bernardo D, Omar Al-Hassi H, Landy J, Peake ST, Thomas LV, Tee CT, Lee GH, Hart AL, Yaqoob P, Knight SC. Dysregulated circulating dendritic cell function in ulcerative colitis is partially restored by probiotic strain *Lactobacillus casei* Shirota. Mediators Inflamm. 2013; 2013:573–576.

77. Mann ER, Bernardo D, Ng SC., Rigby RJ, Al-Hassi HO, Landy J, Peake STC, Spranger H, English NR, Thomas LV, Stagg AJ, Knight SC, Hart AL. Human gut dendritic cells drive aberrant gut-specific T-cell responses in ulcerative colitis, characterized by increased IL-4 production and loss of IL-22 and IFN-γ Inflamm. Bowel Dis. 2014; 20(12):2299–2307.

78. Gómez-Llorente C, Muñoz S, Gil A. Role of toll-like receptors in the development of immunotolerance mediated by probiotics. Proc. Nutr. Soc. 2010; 69(3):381–389.

79. Wu Y, Zhu C, Chen Z, Chen Z, Zhang W, Ma X, Wang L, Yang X, Jiang Z. Protective effects of *Lactobacillus plantarum* on epithelial barrier disruption caused by enterotoxigenic *Escherichia coli* in intestinal porcine epithelial cells. Vet. Immunol. Immunopathol. 2016; 172:55–63.

80. Wachi S, Kanmani P, Tomosada Y, Kobayashi H, Yuri T, Egusa S, Shimazu T, Suda Y, Aso H, Sugawara M, Saito T, Mishima T, Villena J, Kitazawa H. *Lactobacillus delbrueckii* TUA4408L and its extracellular polysaccharides attenuate enterotoxigenic *Escherichia coli*-induced inflammatory response in porcine intestinal epitheliocytes via Toll-like receptor-2 and 4. Mol. Nutr. Food Res. 2014; 58(10):2080–2093.

81. Prisciandaro LD, Geier MS, Chua AE, Butler RN, Cummins AG, Sander GR, Howarth GS. Probiotic factors partially prevent changes to caspases 3 and 7 activation and transepithelial electrical resistance in a model of 5-fluorouracil-induced epithelial cell damage. Support Care Cancer. 2012; 20(12):3205–3210.

82. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ, Romero F, Gil A. Human intestinal dendritic cells decrease cytokine release against Salmonella infection in the presence of *Lactobacillus paracasei* upon TLR activation. PLoS ONE. 2012; 7(8):e43197.

83. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ, Romero F, Gil A. Cell-free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases pro-inflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation. PLoS ONE. 2013; 8(3):e59370.

84. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Romero F, Gil A. *Lactobacillus rhamnosus* and its cell-free culture supernatant differentially modulate inflammatory biomarkers in *Escherichia coli*-challenged human dendritic cells. Br. J. Nutr. 2014; 111(10):1727–1737.

85. Abdelouhab K, Rafa H, Toumi R, Bouaziz S, Medjeber O, Touil-Boukoffa C. Mucosal intestinal alteration in experimental colitis correlates with nitric oxide production by peritoneal macrophages: Effect of probiotics and prebiotics. Immunopharmacol. Immunotoxicol. 2012; 34(4):590–597.

86. Atkins HL, Geier MS, Prisciandaro LD, Pattanaik AK, Forder RE, Turner MS, Howarth GS. Effects of a *Lactobacillus reuteri* BR11 mutant deficient in the cystine-transport system in a rat model of inflammatory bowel disease. Dig. Dis. Sci. 2012; 57(3):713–719.

87. Cui Y, Wei H, Lu F, Liu X, Liu D., Gu L, Ouyang C. Different effects of three selected *Lactobacillus* strains in dextran sulfate sodium-induced colitis in BALB/c mice. PLoS ONE. 2016; 11(2):e0148241.

88. Wong CC, Zhang L, Li ZJ, Wu WK, Ren SX, Chen YC, Ng TB, Cho CH. Protective effects of cathelicidin-encoding *Lactococcus lactis* in murine ulcerative colitis. J. Gastroenterol. Hepatol. 2012; 27(7):1205–1212.

89. Hong YS, Ahn YT, Park JC, Lee JH, Lee H, Huh CS, Kim DH, Ryu DH, Hwang GS. 1H NMR-based metabonomic assessment of probiotic effects in a colitis mouse model. Arch. Pharm. Res. 2010; 33(7):1091–1101

90. Garrido-Mesa N, Utrilla P, Comalada M, Zorrilla P, Garrido-Mesa J., Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. The association of minocycline and the probiotic *Escherichia coli* Nissle 1917 results in an additive beneficial effect in a DSS model of reactivated colitis in mice. Biochem. Pharmacol. 2011; 82(12):1891–1900.

91. Zheng B, van Bergenhenegouwen J, van de Kant HJ, Folkerts G, Garssen J, Vos AP, Morgan ME, Kraneveld AD. Specific probiotic dietary supplementation leads to different effects during remission and relapse in murine chronic colitis. Benef. Microbes. 2016; 7(2):205–213.

92. Talero E, Bolivar S, Ávila-Román J, Alcaide A, Fiorucci S, Motilva V. Inhibition of chronic ulcerative colitis-associated adenocarcinoma development in mice by VSL#3. Inflamm. Bowel. Dis. 2015; 21(5):1027–1037.

93. Dai C, Zheng CQ, Meng FJ, Zhou Z, Sang LX, Jiang M. VSL#3 probiotics exerts the anti-inflammatory activity via PI3k/Akt and NF-κB pathway in rat model of DSS-induced colitis. Mol. Cell Biochem. 2013; 374(1-2):1–11.

94. Salim SY, Young PY, Lukowski CM, Madsen KL, Sis B, Churchill TA, Khadaroo RG. VSL#3 probiotics provide protection against acute intestinal ischaemia/reperfusion injury. Benef. Microbes. 2013; 4(4):357–365.

95. Zhang HL, Li S, Xu DN, Zheng WW, Liu Y, Chen J, Qiu ZB, Dorfman RG, Zhang J, Liu J. Mucosa-reparing and microbiota-balancing therapeutic effect of *Bacillus subtilis* alleviates dextrate sulfate sodium-induced ulcerative colitis in mice. Exp. Ther. Med. 2016; 12(4):2554–2562.

96. Satish Kumar C, Kondal Reddy K, Reddy AG, Vinoth A, Ch SR, Boobalan G, Rao GS. Protective effect of *Lactobacillus plantarum* 21, a probiotic on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats. Int. Immunopharmacol. 2015; 25(2):504–510.

97. Yang F, Wang A, Zeng X, Hou C, Liu H, Qiao S. *Lactobacillus reuteri* I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. BMC Microbiol. 2015; 15:32.

98. Eeckhaut V, Machiels K, Perrier C, Romero C, Maes S, Flahou B, Steppe M, Haesebrouck F, Sas B, Ducatelle R, Vermeire S, Van Immerseel F. *Butyricicoccus pullicaecorum* in inflammatory bowel disease. Gut. 2013; 62:1745–1752.

99. Schmitz S, Werling D, Allenspach K. Effects of ex-vivo and in-vivo treatment with probiotics on the inflammasome in dogs with chronic enteropathy. PLoS ONE. 2015; 10(3):e0120779.

100. Sun Q, Shi Y, Wang F, Han D, Lei H, Zhao Y, Sun Q. Study on the effects of microencapsulated *Lactobacillus delbrueckii* on the mouse intestinal flora. J. Microencapsul. 2015; 32(7):669–676.

101. Štofilová J, Szabadosová V, Hrčková G, Salaj R, Bertková I, Hijová E, Strojný L, Bomba A. Co-administration of a probiotic strain *Lactobacillus plantarum* LS/07 CCM7766 with prebiotic

inulin alleviates the intestinal inflammation in rats exposed to N,N-dimethylhydrazine. Int. Immunopharmacol. 2015; 24:361–368.

102. Ogita T, Bergamo P, Maurano F, D′Arienzo R, Mazzarella G, Bozzella G, Luongo D, Sashihara T, Suzuki T, Tanabe S, Rossi M. Modulatory activity of *Lactobacillus rhamnosus* OLL2838 in a mouse model of intestinal immunopathology. Immunobiology. 2015; 220(6):701–710.

103. Wu S, Yoon S, Zhang YG, Lu R, Xia Y, Wan J, Petrof EO, Claud EC, Chen D, Sun J. Vitamin D receptor pathway is required for probiotic protection in colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 2015; 309(5):G341–G349.

104. Tamaki H, Nakase H, Inoue S, Kawanami C, Itani T, Ohana M, Kusaka T, Uose S, Hisatsune H, Tojo M, Noda T, Arasawa S, Izuta M, Kubo A, Ogawa C, Matsunaka T, Shibatouge M. Efficacy of probiotic treatment with *Bifidobacterium longum* 536 for induction of remission in active ulcerative colitis: A randomized, double-blinded, placebo-controlled multicenter trial. Dig. Endosc. 2016; 28(1):67–74.

105. Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, Aoki H, Tsuda Y, Hosoe N, Takada N, Suzuki Y. Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis. World J. Gastroenterol. 2015; 21(19):5985–5994.

106. Krag A, Munkholm P, Israelsen H, von Ryberg B, Andersen KK, Bendtsen F. Profermin is efficacious in patients with active ulcerative colitis-a randomized controlled trial. Inflamm. Bowel Dis. 2013; 19(12):2584–2592.

107. Petersen AM, Mirsepasi H, Halkjær SI, Mortensen EM, Nordgaard-Lassen I, Krogfelt KA. Ciprofloxacin and probiotic *Escherichia coli* Nissle add-on treatment in active ulcerative colitis: A double-blind randomized placebo controlled clinical trial. J. Crohns Colitis. 2014; 8(11):1498–1505.

108. Fedorak RN, Feagan BG, Hotte N, Leddin D, Dieleman LA, Petrunia DM, Enns R, Bitton A, Chiba N, Paré P. The probiotic VSL#3 has anti-inflammatory effects and could reduce endoscopic recurrence after surgery for crohn's disease. Clin. Gastroenterol. Hepatol. 2015; 13(5):928–935.

109. Chia-Hui Yu L, Wei SC, and Ni YH. Interplay between the gut microbiota and epithelial innate signaling in colitis-associated colon carcinogenesis Cancer Research Frontiers. 2017; 3(1):1-28.

110. Zackular JP, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, Schloss PD. The gut microbiome modulates colon tumorigenesis. MBio. 2013; 4(6):e00692-13.

111. Wu S Rhee KJ, Albesiano E, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat. Med. 2009; 15(9):1016–1022.

112. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. T reg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013; 500(7461):232–236.

113. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc. Natl. Acad. Sci. USA. 2014; 111(6):2247–2252.

114. Chang JH, Shim Y., Cha SK, Reaney MJT, Chee KM. Effect of *Lactobacillus acidophilus* KFRI342 on the development of chemically induced precancerous growths in the rat colon. J. Med. Microbiol. 2012; 61(Pt 3):361–368.

115. Zhu J, Zhu C, Ge S, Zhang M, Jiang L, Cui J, Ren F. *Lactobacillus salivarius* Ren prevent the early colorectal carcinogenesis in 1,2-dimethylhydrazine-induced rat model. J. Appl. Microbiol. 2014; 117(1):208–216.

116. Zhang M, Fan X, Fang B, Zhu C, Zhu J, Ren F. Effects of *Lactobacillus salivarius* Ren on cancer prevention and intestinal microbiota in 1, 2-dimethylhydrazine-induced rat model. J. Microbiol. 2015; 53(6):398–405.333

117. Lee HA, Kim H, Lee K-W, Park K-Y. Dead nano-sized *Lactobacillus plantarum* inhibits azoxymethane/dextran sulfatesodium–induced colon cancer in Balb/c mice. J. Med. Food. 2015; 18(12):1400–1405.

118. Gagniere J, Raisch J, Veziant J, Barnich N, Bonnet R, Buc E, Bringer M-A, Pezet D, Bonnet M. Gut microbiota imbalance and colorectal cancer. World J. Gastroenterol. 2016; 22(2):501–518.

119. Chen CC, Lin WC, Kong MS, Shi HN, Walker WA, Lin CY, Huang CT, Lin YC, Jung SM, Lin TY. Oral inoculation of probiotics *Lactobacillus acidophilus* NCFM suppresses tumour growth both in segmental orthotopic colon cancer and extra-intestinal tissue. Br. J. Nutr. 2012; 107(11):1623–1634.

120. Baxter NT, Zackular JP, Chen GY, Schloss PD. Structure of the gut microbiome following colonization with human feces determines colonic tumor burden. Microbiome. 2014; 2:20.

121. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, Honda K. Induction of colonic regulatory T cells by indigenous Clostridium species. Science. 2011; 331(6015):337–341.

122. Evrard B, Coudeyras S, Dosgilbert A, Charbonnel N, Alamé J, Tridon A, Forestier C. Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35. PLoS ONE. 2011; 6(4):e18735.

123. Kuugbee ED, Shang X, Gamallat Y, Bamba D, Awadasseid A, Suliman MA, Zang S, Ma Y, Chiwala G, Xin Y, Shang D. Structural change in microbiota by a probiotic cocktail enhances the gut barrier and reduces cancer via TLR2 signaling in a rat model of colon cancer. Dig. Dis. Sci. 2016; 61(10):2908–2920.

124. Gamallat Y, Meyiah A, Kuugbee ED, Hago AM, Chiwala G, Awadasseid A, Bamba D, Zhang X, Shang X, Luo F, Xin Y. *Lactobacillus rhamnosus* induced epithelial cell apoptosis, ameliorates inflammation and prevents colon cancer development in an animal model. Biomed. Pharmacother. 2016; 83:536–541.

125. del Carmen S, deMoreno de LeBlanc A, Levit R, Azevedo V, Langella P, Bermúdez-Humarán LG, Guy LeBlanc J. Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer mouse model. Int. Immunopharm. 2017; 42:122–129.

126. Verma A, Shukla G. Probiotics *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* suppresses DMH-induced procarcinogenic fecal enzymes and preneoplastic aberrant crypt foci in early colon carcinogenesis in Sprague Dawley rats. Nutr. Cancer. 2013; 65(1):84–91.

127. Zhu Q, Gao R, Wu W, Qin H. The role of gut microbiota in the pathogenesis of colorectal cancer. Tumor Biol. 2013; 34(3):1285–1300.

128. Ewaschuk JB, Walker JW, Diaz H, Madsen KL. Bioproduction of conjugated linoleic acid by probiotic bacteria occurs *in vitro* and *in vivo* in mice. J. Nutr. 2006; 136:1483–1487.

129. Hatakka K, Holma R, El-Nezami H, Suomalainen T, Kuisma M, Saxelin M, Poussa T, Mykkänen H, Korpela R. The influence of *Lactobacillus rhamnosus* LC705 together with *Propionibacterium freudenreichii ssp. shermanii JS* on potentially arcinogenic bacterial activity in human colon. Int. J. Food Microbiol. 2008; 128(2):406–410.

130. Ohara T, Yoshino K, Kitajima M. Possibility of preventing colorectal carcinogenesis with probiotics. Hepatogastroenterology. 2010; 57(104):1411–1415.

131. Liu Z, Qin H, Yang Z, Xia Y, Liu W, Yang J, Jiang Y, Zhang H, Yang Z, Wang Y, Zheng Q. Randomised clinical trial: The effects of perioperative probiotic treatment on barrier function and post-operative infectious complications in colorectal cancer surgery: A double-blind study. Aliment Pharmacol. Ther. 2011; 33(1):50–63.

132. Vinderola G, Perdigón G, Duarte J, Farnworth E, Matar C. Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. Cytokine. 2006; 36(5-6):254–260.

133. Galdeano CM, Perdigón G. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin. Vaccine Immunol. 2007; 13(2):219–226.

134. Karczewski J, Troost FJ, Konings I, Dekker J, Kleerebezem M, Brummer RJ, Wells JM. Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum in vivo* and protective effects on the epithelial barrier. Am J Physiol Gastrointest Liver Physiol. 2010; 298(6):G851–G859.

135. Madsen KL. Enhancement of epithelial barrier function by probiotics. J. Epithel. Biol. Pharmacol. 2012; 5:55–59.

136. LeBlanc AM, Perdigón G. Yogurt feeding inhibits promotion and progression of experimental colorectal cancer. Med. Sci. Monit. 2004; 10(4):96–104.

137. Wan Y, Xin Y, Zhang C, Wu ., Ding D, Tang L, Owusu L, Bai J, Li W. Fermentation supernatants of *Lactobacillus delbrueckii* inhibit growth of human colon cancer cells and induce apoptosis through a caspase 3–dependent pathway. Oncol. Lett. 2014; 7(5):1738–1742.

138. Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M O'Sullivan GC, Pool-Zobel P, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Van Loo J, Watzl B, Collins JK. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am. J. Clin. Nutr. 2007; 85(2):488–496.

139. Kotzampassi K, Stavrou G, Damoraki G, Georgitsi M, Basdanis G, Tsaousi G, Giamarellos-Bourboulis EJ. A four-probiotics regimen reduces postoperative complications after colorectal surgery: A randomized, double-blind, placebo-controlled study. World J. Surg. 2015; 39(11):2776–2783.

140. Pala V, Sieri S, Berrino F, Vineis P, Sacerdote C, Palli D, Masala G, Panico S, Mattiello A, Tumino R, Giurdanella MC, Agnoli C, Grioni S, Krogh V. Yogurt consumption and risk of colorectal cancer in the Italian European prospective investigation into cancer and nutrition cohort. Int. J. Cancer. 2011; 129(11):2712–2719.

141. Koliarakis I, Psaroulaki A, Nikolouzakis TK, Sgantzos MN, Goulielmos G, Androutsopoulos VP, Tsiaoussis J, Tsatsakis A, Kokkinakis M. Intestinal microbiota and colorectal cancer: A new aspect of research. J. BUON. 2018; 23(5):1216–1234.

142. Roy S, Trinchieri G. Microbiota: A key orchestrator of cancer therapy. Nat. Rev. Cancer. 2017; 17(5):271–285.

143. Zitvogel L, Ma Y, Raoult D, Kroemer G, Gajewski TF. The microbiome in cancer immunotherapy: Diagnostic tools and therapeutic strategies. Science. 2018; 359(6382):1366–1370.

144. Di Marzio L, Cinque B, De Simone C, Cifone MG. Effect of the lactic acid bacterium *Streptococcus thermophilus* on ceramide levels in human keratinocytes *in vitro* and stratum corneum *in vivo*. J. Invest. Dermatol. 1999; 113(1):98-106.

145. Di Marzio L, Centi C, Cinque B, Masci S, Giuliani M, Arcieri A, Zicari L, De Simone C, Cifone MG. Effect of the lactic acid bacterium *Streptococcus thermophilus* on stratum corneum ceramide levels and signs and symptoms of atopic dermatitis patients. Exp. Dermatol. 2003; 12(5):615-620.

146. Di Marzio L, Cinque B, Cupelli F, De Simone C, Cifone MG, Giuliani M. Increase of skin-ceramide levels in aged subjects following a short-term topical application of bacterial sphingomyelinase from *Streptococcus thermophilus*. Int. J. Immunopathol. Pharmacol. 2008; 21(1):137-143.

147. Guéniche A, Cathelineau AC, Bastien P, Esdaile J, Martin R, Queille Roussel C, Breton L. *Vitreoscilla filiformis* biomass improves seborrheic dermatitis. J. Eur. Acad. Dermatol. Venereol. 2008; 22(8):1014-1015.

148. Kang BS, Seo JG, Lee GS, Kim JH, Kim SY, Han YW, Kang H, Kim HO, Rhee JH, Chung MJ, Park YM. Antimicrobial activity of enterocins from *Enterococcus faecalis* SL-5 against *Propionibacterium acnes*, the causative agent in acne vulgaris, and its therapeutic effect. J. Microbiol. 2009; 47(1):101-109.

149. Peral MC, Martinez MA, Valdez JC. Bacteriotherapy with *Lactobacillus plantarum* in burns. Int. Wound J. 2009; 6(1):73-81.

150. Guéniche A, Bastien P, Ovigne JM, Kermici M, Courchay G, Chevalier V, Breton L, Castiel-Higounenc I. *Bifidobacterium longum* lysate, a new ingredient for reactive skin. Exp. Dermatol. 2010; 19(8):e1-8.

151. Peral MC, Rachid MM, Gobbato NM, Huaman Martinez MA, Valdez JC. Interleukin-8 production by polymorphonuclear leukocytes from patients with chronic infected leg ulcers treated with *Lactobacillus plantarum*. Clin Microbiol Infect. 2010; 16(3):281–6.

152. Muizzuddin N, Maher W, Sullivan M, Schnittger S, Mammone T. Physiological effect of a probiotic on skin. J Cosmet Sci. 2012; 63(6):385-395.

153. Zoccali G, Cinque B, La Torre C, Lombardi F, Palumbo P, Romano L, Mattei A, Orsini G, Cifone MG, Giuliani M. Improving the outcome of fractional CO2 laser resurfacing using a probiotic skin cream: Preliminary clinical evaluation. Lasers Med. Sci. 2016; 31(8):1607-1611.

154. Blanchet-Réthoré S, Bourdès V, Mercenier A, Haddar CH Verhoeven PO, Andres P. Effect of a lotion containing the heat-treated probiotic strain *Lactobacillus johnsonii* NCC 533 on *Staphylococcus aureus* colonization in atopic dermatitis. Clin. Cosmet. Investig. Dermatol. 2017; 10:249–257.

155. Nakatsuji T, Chen TH, Narala S, Chun KA, Two AM, Yun T, Shafiq F, Kotol PF, Bouslimani A, Melnik AV, Latif H, Kim JN, Lockhart A, Artis K, David G, Taylor P, Streib J, Dorrestein PC, Grier A, Gill SR, Zengler K, Hata TR, Leung DY, Gallo RL. Antimicrobials from human skin commensal bacteria protect against *Staphylococcus aureus* and are deficient in atopic dermatitis. Sci. Transl. Med. 2017; 9(378):eaah4680.

156. Twetman S, Pedersen AML, Yucel-Lindberg T. Probiotic supplements containing *Lactobacillus reuteri* does not affect the levels of matrix metalloproteinases and interferons in oral wound healing. BMC Res. Notes. 2018; 11(1):759.

157. Lee J, Yang W, Hostetler A, Schultz N, Suckow MA, Stewart KL, Kim DD, Kim HS. Characterization of the anti-inflammatory *Lactobacillus reuteri* BM36301 and its probiotic benefits on aged mice. BMC Microbiol. 2016; 16:69–83.

158. Wang Y, Kao MS, Yu J, Huang S, Marito S, Gallo RL, Huang CM. A precision microbiome approach using Sucrose for selective augmentation of *Staphylococcus epidermidis* fermentation against *Propionibacterium acnes*. Int. J. Mol. Sci. 2016; 17(11):1870.

159. Holz C, Benning J, Schaudt M, Heilmann A, Schultchen J, Goelling D, Lang C. Novel bioactive from *Lactobacillus brevis* DSM17250 to stimulate the growth of *Staphylococcus epidermidis*: A pilot study. Benef. Microbes. 2017; 8(1):121–131.

160. Al-Ghazzewi FH, Tester RF. Impact of prebiotics and probiotics on skin health. Benef Microbes. 2014; 5(2):99–107.

161. Oh S, Kim SH, Ko Y, Sim JH, Kim KS, Lee SH, Park S, Kim YJ. Effect of bacteriocin produced by *Lactococcus sp*. HY 449 on skin-inflammatory bacteria. Food Chem. Toxicol. 2006; 44:1184–1190.

162. Manzhalii E, Hornuss D, Stremmel W. Intestinal-borne dermatoses significantly improved by oral application of *Escherichia coli Nissle 1917*. World J. Gastroenterol. 2016; 22(23):5415–5421.

163. Fabbrocini G, Bertona M, Picazo O, Pareja-Galeano H, Emanuele E. Supplementation with *Lactobacillus rhamnosus* SP1 normalises skin expression of genes implicated in insulin signalling and improves adult acne. Benef. Microbes. 2016; 7(5):625–630.

164. Hawkes JE, Chan TC, Krueger JG. Psoriasis pathogenesis and the development of novel targeted immune therapies. J. Allergy Clin. Immunol. 2017; 140(3):645–653.

165. Chen YH, Wu CS, Chao YH, Lin CC, Tsai HY, Li YR, Chen YZ, Tsai WH, Chen YK. *Lactobacillus pentosus* GMNL-77 inhibits skin lesions in imiquimod-induced psoriasis-like mice. J. Food Drug Anal. 2017; 25(3):559–566.

166. Satta R, Pes GM, Rocchi C, Pes MC, Dore MP. Is probiotic use beneficial for skin lesions in patients with inflammatory bowel disease? J. Dermatolog. Treat. 2019; 30(6):612-616.

167. Kim JA, Kim SH, Kim IS, Yu DY, Kim SC, Lee S.H., Lee S.S., Yun C.H., Choi I.S., Cho K.K. Anti-Inflammatory Effects of a Mixture of Lactic Acid Bacteria and Sodium Butyrate in Atopic Dermatitis Murine Model. J. Med. Food. 2018; 21(7):716–725.

168. Prakoeswa CRS, Herwanto N, Prameswari R, Astari L, Sawitri S, Hidayati AN, Indramaya DM, Kusumowidagdo ER, Surono IS. *Lactobacillus plantarum* IS-10506 supplementation reduced SCORAD in children with atopic dermatitis. Benef. Microbes. 2017; 8(5):833–840.

169. Inoue Y, Kambara T, Murata N, Komori-Yamaguchi J, Matsukura S, Takahashi Y, Ikezawa Z, Aihara M. Effects of oral administration of *Lactobacillus acidophilus* L-92 on the symptoms and serum cytokines of atopic dermatitis in Japanese adults: A double-blind, randomized, clinical trial. Int. Arch. Allergy Immunol. 2014; 165(4):247–254.

179. Holowacz S, Blondeau C, Guinobert I, Guilbot A, Hidalgo S, Bisson JF. *Lactobacillus salivarius* LA307 and *Lactobacillus rhamnosus* LA305 attenuate skin inflammation in mice. Benef. Microbes. 2018; 9(2):299–309.

171. Choi WJ, Konkit M, Kim Y, Kim MK, Kim W. Oral administration of *Lactococcus chungangensis* inhibits 2,4-dinitrochlorobenzene-induced atopic-like dermatitis in NC/Nga mice. J. Dairy Sci. 2016; 99(9):6889–6901.

172. Lee SH, Yoon JM, Kim YH, Jeong DG, Park S, Kang DJ. Therapeutic effect of tyndallized *Lactobacillus rhamnosus* IDCC 3201 on atopic dermatitis mediated by down-regulation of immunoglobulin E in NC/Nga mice. Microbiol. Immunol. 2016; 60(7):468–476.

173. Rosignoli C, Thibaut de Ménonville S, Orfila D, Béal M, Bertino B Aubert J, Mercenier A, Piwnica D. A topical treatment containing heat-treated *Lactobacillus johnsonii* NCC 533 reduces *Staphylococcus aureus* adhesion and induces antimicrobial peptide expression in an *in vitro* reconstructed human epidermis model. Exp. Dermatol. 2018; 27(4):358–365.

174. Sharma D, Kober MM, Bowe WP. Anti-aging effects of probiotics. J. Drugs Dermatol. 2016; 15(1):9–12.

175. Im AR, Kim HS, Hyun JW, Chae S. Potential for tyndalized *Lactobacillus acidophilus* as an effective component in moisturizing skin and anti-wrinkle products. Exp. Ther. Med. 2016; 12(2):759–64.

176. Satoh T., Murata M., Iwabuchi N., Odamaki T., Wakabayashi H., Yamauchi K., Abe F., Xiao J.Z. Effect of *Bifidobacterium breve* B-3 on skin photoaging induced by chronic UV irradiation in mice. Benef. Microbes. 2015; 6(4):497–504.

177. Bouilly-Gauthier C, Maubert JY, Duteil L, Queille-Roussel C, Piccardi N, Montastier C, Manissier P, Pierard G, Ortonne JP. Clinical evidence of benefits of a dietary supplement containing probiotic and carotenoids on ultraviolet-induced skin damage. Br. J. Dermatol. 2010; 163(3):536–543.

178. Mahé YF, Martin R, Aubert L, Billoni N Collin C, Pruche F, Bastien P Drost SS, Lane AT, Meybeck A. Induction of the skin endogenous protective mitochondrial MnSOD by *Vitreoscilla filiformis* extract. Int. J. Cosmet. Sci. 2006; 28(4):277–287.

179. Park MJ, Bae YS. Fermented acanthopanax koreanum root extract reduces UVB- and H_2O_2-induced senescence in human skin fibroblast cells. J. Microbiol. Biotechnol. 2016; 26(7):1224–1233.

180. Weinstein C, Ramirez OM, Pozner JN. Postoperative care following CO_2 laser resurfacing: Avoiding pitfalls. Plast. Reconstr. Surg. 1997; 100(7):1855–1866.

181. Cinque B, La Torre C, Melchiorre E, Marchesani G, Zoccali G, Palumbo P, Di Marzio L, Masci A, Mosca L, Mastromarino P, Giuliani M, Cifone MG. Use of Probiotics for Dermal Applications. Probiotics. In: Min-Tze Liong. Microbiology Monographs. 2011; vol. 21, pp. 221-241.

182. Jones M, Ganopolsky JG, Labbé A, Gilardino M, Wahl C, Martoni C, Prakash S. Novel nitric oxide producing probiotic wound healing patch: Preparation and *in vivo* analysis in a New Zealand white rabbit model of ischaemic and infected wounds. Int. Wound J. 2012; 9(3):330–343.

183. Mohammedsaeed W, Cruickshank S McBain AJ, O'Neill CA. *Lactobacillus rhamnosus* GG lysate increases reepithelialization of keratinocyte scratch assays by promoting migration. Sci. Rep. 2015; 5:16147.

184. Sultana R, McBain AJ, O'Neill CA. Strain-dependent augmentation of tight-junction barrier function in human primary epidermal keratinocytes by *Lactobacillus* and *Bifidobacterium* lysates. Appl. Environ. Microbiol. 2013; 79(16):4887–4894.

185. Lopes EG, Moreira DA, Gullón P, Gullón B, Cardelle-Cobas A, Tavaria FK. Topical application of probiotics in skin: Adhesion, antimicrobial and antibiofilm *in vitro* assays. J. Appl. Microbiol. 2017; 122(2):450–461.

186. Iwase T, Uehara Y, Shinji H, Tajima A, Seo H, Takada K, Agata T, Mizunoe Y. *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. Nature. 2010; 465(7296):346–349.

187. Taverniti V, Guglielmetti S. The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: Proposal of paraprobiotic concept). Genes Nutr. 2011; 6(3):261–274.

188. Kimoto-Nira H, Suzuki C, Kobayashi M, Sasaki K, Kurisaki J, Mizumachi K. Anti-ageing effect of a lactococcal strain: Analysis using senescence-accelerated mice. Br. J. Nutr. 2007; 98(6):1178–1186.

189. Li H, Limenitakis JP, Fuhrer T, Geuking MB, Lawson MA, Wyss M, Brugiroux S, Keller I, Macpherson JA, Rupp S, Stolp B, Stein JV, Stecher B, Sauer U, McCoy KD, Macpherson AJ. The outer mucus layer hosts a distinct intestinal microbial niche. Nat. Commun. 2015; 6:8292.

190. Tejero-Sariñena S, Barlow J, Costabile A, Gibson GR, Rowland I. *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. Anaerobe. 2012; 18(5):530–538.

191. Atassi F, Brassart D, Grob P, Graf F, Servin AL. *Lactobacillus* strains isolated from the vaginal microbiota of healthy women inhibit *Prevotella bivia* and *Gardnerella vaginalis* in coculture and cell culture. FEMS Immunol. Med. Microbiol. 2006; 48(3):424–432.

192. Breshears LM, Edwards VL, Ravel J, Peterson ML. *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. BMC Microbiol. 2015; 15:276.

193. Kang DH, Fung DY. Effect of diacetyl on controlling *Escherichia coli* O157:H7 and *Salmonella Typhimurium* in the presence of starter culture in a laboratory medium and during meat fermentation. J. Food Prot. 1999; 62(9):975–9.

194. Langa S, Martín-Cabrejas I, Montiel R, Landete JM, Medina M, Arqués JL. Short communication: Combined antimicrobial activity of reuterin and diacetyl against foodborne pathogens. J. Dairy Sci. 2014; 97(10):6116–6121.

195. Schaefer L, Auchtung TA, Hermans KE, Whitehead D, Borhan B, Britton RA. The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. Microbiology. 2010; 156(Pt 6):1589–1599.

196. Arqués JL, Rodríguez E, Nuñez M, Medina M. Antimicrobial activity of nisin, reuterin, and the lactoperoxidase system on *Listeria monocytogenes* and *Staphylococcus aureus* in cuajada, a semisolid dairy product manufactured in Spain. J. Dairy Sci. 2008; 91(1):70–75.

197. Pascual LM, Daniele MB, Giordano W, Pájaro MC, Barberis IL. Purification and partial characterization of novel bacteriocin L23 produced by *Lactobacillus fermentum* L23. Curr. Microbiol. 2008; 56(4):397–402.

198. Kim SY, Shin S, Koo HC, Youn JH, Paik HD, Park YH. *In vitro* antimicrobial effect and *in vivo* preventive and therapeutic effects of partially purified lantibiotic lacticin NK34 against infection by *Staphylococcus* species isolated from bovine mastitis. J. Dairy Sci. 2010; 93(8):3610–3615.

199. Jena PK, Trivedi D, Chaudhary H, Sahoo TK, Seshadri S. Bacteriocin PJ4 active against enteric pathogen produced by *Lactobacillus helveticus* PJ4 isolated from gut microflora of wistar rat (*Rattus norvegicus*): Partial purification and characterization of bacteriocin. Appl. Biochem. Biotechnol. 2013; 169(7):2088–2100.

200. Zakaria Gomaa E. Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability. J. Gen Appl. Microbiol. 2013; 59(6):425–436.

201. Santos CM, Pires MC, Leão TL, Hernández ZP, Rodriguez ML, Martins AKS, Miranda LS, Martins FS, Nicoli JR. Selection of *Lactobacillus* strains as potential probiotics for vaginitis treatment. Microbiology. 2016; 162(7):1195–1207.

202. Živković M, Miljković MS, Ruas-Madiedo P, Markelić MB, Veljović K, Tolinački M, Soković S, Korać A, Golić N. EPS-SJ Exopolisaccharide Produced by the strain *Lactobacillus paracasei subsp. paracasei* BGSJ2-8 is involved in adhesion to epithelial intestinal cells and decrease on *E. coli* association to Caco-2 Cells. Front Microbiol. 2016; 7:286.

203. Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb. Perspect. Med. 2012; 2(11):a012427.

204. Pastar I, Nusbaum AG, Gil J, Patel SB, Chen J, Valdes J, Stojadinovic O, Plano LR, Tomic-Canic M, Davis SC. Interactions of methicillin resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in polymicrobial wound infection. PLoS One. 2013; 8(2):e56846.

205. Sully EK, Malachowa N, Elmore BO, Alexander SM, Femling JK, Gray BM, DeLeo FR, Otto M, Cheung AL, Edwards BS, Sklar LA, Horswill AR, Hall PR, Gresham HD. Selective chemical inhibition of agr quorum sensing in *Staphylococcus aureus* promotes host defense with minimal impact on resistance. PLoS Pathog. 2014; 10(6):e1004174.

206. Kim HS, Lee SH, Byun Y, Park HD. 6-Gingerol reduces *Pseudomonas aeruginosa* biofilm formation and virulence via quorum sensing inhibition. Sci Rep. 2015; 5:8656.

207. Valdéz JC, Peral MC, Rachid M, Santana M, Perdigón G. Interference of *Lactobacillus plantarum* with *Pseudomonas aeruginosa in vitro* and in infected burns: the potential use of probiotics in wound treatment. Clin. Microbiol. Infect. 2005; 11(6):472–479.

208. Liévin-Le Moal V, Servin AL. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin. Microbiol. Rev. 2006; 19(2):315–337.

209. Anderson RC, Cookson AL, McNabb WC, Park Z, McCann MJ, Kelly WJ, Roy NC. *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol. 2010; 10:316.

210. Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F. Probiotics promote gut health through stimulation of epithelial innate immunity. Proc. Natl. Acad. Sci. USA. 2010; 107(1):454–459.

211. Ewaschuk JB, Diaz H, Meddings L, Diederichs B, Dmytrash A, Backer J, Looijer-van Langen M, Madsen KL. Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function. Am. J. Physiol. Gastrointest. Liver Physiol. 2008; 295(5):G1025–G1034.

212. Alam A, Leoni G, Wentworth CC, Kwal JM, Wu H, Ardita CS, Swanson PA, Lambeth JD, Jones RM, Nusrat A, Neish AS. Redox signaling regulates commensal-mediated mucosal homeostasis and restitution and requires formyl peptide receptor 1. Mucosal Immunol. 2014; 7(3):645–655.

213. Nasrabadi MH, Aboutalebi H, Ebrahimi MT, Zahedi F. The healing effect *of Lactobacillus plantarum* isolated from Iranian traditional cheese on gastric ulcer in rats. African Journal of Pharmacy and Pharmacology. 2011:1446–51.

214. Pastar I, Stojadinovic O, Yin NC, Ramirez H, Nusbaum AG, Sawaya A, Patel SB, Khalid L, Isseroff RR, Tomic-Canic M. Epithelialization in wound healing: A Comprehensive Review. Adv. Wound Care (New Rochelle) 2014; 3(7):445–464.

215. Schlee M, Harder J, Köten B, Stange EF, Wehkamp J, Fellermann K. Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clin. Exp. Immunol. 2008; 151(3):528–535.

216. Otte JM, Werner I, Brand S, Chromik AM, Schmitz F, Kleine M, Schmidt WE. Human beta defensin 2 promotes intestinal wound healing *in vitro*. J. Cell. Biochem. 2008; 104(6):2286–2297.

217. Dharmani P, De Simone C, Chadee K. The probiotic mixture VSL#3 accelerates gastric ulcer healing by stimulating vascular endothelial growth factor. PLoS One. 2013; 8(3):e58671.

218. Fordjour L, D'Souza A, Cai C, Ahmad A, Valencia G, Kumar D, Aranda JV, Beharry KD. Comparative effects of probiotics, prebiotics, and synbiotics on growth factors in the large bowel in a rat model of formula-induced bowel inflammation. J. Pediatr. Gastroenterol. Nutr. 2010; 51(4):507–513.

219. Wang L, Cao H, Liu L, Wang B, Walker WA, Acra SA, Yan F. Activation of epidermal growth factor receptor mediates mucin production stimulated by p40, a *Lactobacillus rhamnosus* GG-derived protein. J. Biol. Chem. 2014; 289(29):20234–20244.

220. Wang Y, Kirpich I, Liu Y, Ma Z, Barve S, McClain CJ, Feng W. *Lactobacillus rhamnosus* GG treatment potentiates intestinal hypoxia-inducible factor, promotes intestinal integrity and ameliorates alcohol-induced liver injury. Am. J. Pathol. 2011; 179(6):2866–2675.

221. Tu TC, Nagano M, Yamashita T, Hamada H, Ohneda K, Kimura K, Ohneda O. A Chemokine receptor, CXCR4, which is regulated by hypoxia-inducible factor 2α, is crucial for functional endothelial progenitor cells migration to ischemic tissue and wound repair. Stem Cells Dev. 2016; 25(3):266–276.

222. Strbo N, Yin N, Stojadinovic O. Innate and adaptive immune responses in wound epithelialization. Adv. Wound Care (New Rochelle) 2014; 3(7):492–501.

223. Miettinen M, Pietilä TE, Kekkonen RA, Kankainen M, Latvala S, Pirhonen J, Österlund P, Korpela R, Julkunen I. Nonpathogenic *Lactobacillus rhamnosus* activates the inflammasome and antiviral responses in human macrophages. Gut Microbes. 2012; 3(6):510–522.

224. Ren D, Li C, Qin Y, Yin R, Du S, Liu H, Zhang Y, Wang C, Rong F, Jin N. Evaluation of immunomodulatory activity of two potential probiotic *Lactobacillus* strains by *in vivo* tests. Anaerobe. 2015; 35(Pt B):22–7.

225. Kikuchi Y, Kunitoh-Asari A, Hayakawa K, Imai S, Kasuya K, Abe K, Adachi Y, Fukudome S, Takahashi Y, Hachimura S. Oral administration of *Lactobacillus plantarum* strain AYA enhances IgA secretion and provides survival protection against influenza virus infection in mice. PLoS One. 2014; 9(1):e86416.

226. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, Zhou L. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity. 2012; 36(1):92–104.

227. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F, Carvalho A, Puccetti P, Romani L. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013; 39(2):372–385.

228. Takamura T, Harama D, Fukumoto S, Nakamura Y, Shimokawa N, Ishimaru K, Ikegami S, Makino S, Kitamura M, Nakao A. *Lactobacillus bulgaricus* OLL1181 activates the aryl hydrocarbon receptor pathway and inhibits colitis. Immunol. Cell Biol. 2011; 89(7):817–822.

229. Roulis M, Armaka M, Manoloukos M, Apostolaki M, Kollias G. Intestinal epithelial cells as producers but not targets of chronic TNF suffice to cause murine Crohn-like pathology. Proc. Natl. Acad. Sci. USA. 2011; 108(13):5396–3401.

230. Castillo NA, Perdigón G, de Moreno de Leblanc A. Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar Typhimurium infection in mice. BMC Microbiol. 2011; 11:177.

231. Lukic J, Strahinic I, Milenkovic M, Golic N, Kojic M, Topisirovic L, Begovic J. Interaction of *Lactobacillus fermentum* BGHI14 with rat colonic mucosa: implications for colitis induction. Appl. Environ. Microbiol. 2013; 79(18):5735–5744.

232. Ren DY, Li C, Qin YQ, Yin RL, Du SW, Ye F, Liu HF, Wang MP, Sun Y, Li X, Tian MY, Jin NY. Lactobacilli reduce chemokine IL-8 production in response to TNF-α and Salmonella challenge of Caco-2 cells. Biomed. Res. Int. 2013; 2013:925219.

233. Chiba Y, Shida K, Nagata S, Wada M, Bian L, Wang C, Shimizu T, Yamashiro Y, Kiyoshima-Shibata J, Nanno M, Nomoto K. Well-controlled proinflammatory cytokine responses of Peyer's patch cells to probiotic *Lactobacillus casei*. Immunology. 2010; 130(3):352–362.

234. Dong H, Rowland I, Yaqoob P. Comparative effects of six probiotic strains on immune function *in vitro*. Br. J. Nutr. 2012; 108(3):459–470.

235. Takeda K, Suzuki T, Shimada SI, Shida K, Nanno M, Okumura K. Interleukin-12 is involved in the enhancement of human natural killer cell activity by *Lactobacillus casei* Shirota. Clin. Exp. Immunol. 2006; 146(1):109–115.

236. Small CL, McCormick S, Gill N, Kugathasan K, Santosuosso M, Donaldson N, Heinrichs DE, Ashkar A, Xing Z. NK cells play a critical protective role in host defense against acute extracellular Staphylococcus aureus bacterial infection in the lung. J. Immunol. 2008; 180(8):5558–5568.

237. Baquerizo Nole KL, Yim E, Keri JE. Probiotics and prebiotics in dermatology. J. Am. Acad. Dermatol. 2014; 71(4):814–821.

238. Mohammedsaeed W, McBain AJ, Cruickshank SM, O'Neill CA. *Lactobacillus rhamnosus* GG inhibits the toxic effects of *Staphylococcus aureus* on epidermal keratinocytes. Appl Environ Microbiol. 2014; 80(18):5773–5781.

239. Yuki T, Yoshida H, Akazawa Y, Komiya A, Sugiyama Y, Inoue S. Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. J. Immunol. 2011; 187(6):3230–3237.

240. Ramos AN, Cabral ME, Noseda D, Bosch A, Yantorno OM, Valdez JC. Antipathogenic properties of *Lactobacillus plantarum* on *Pseudomonas aeruginosa*: The potential use of its supernatants in the treatment of infected chronic wounds. Wound Repair Regen. 2012; 20(4):552–562.

241. Prince T, McBain AJ, O'Neill CA. *Lactobacillus reuteri* protects epidermal keratinocytes from *Staphylococcus aureus*-induced cell death by competitive exclusion. Appl. Environ. Microbiol. 2012; 78(15):5119–5126.

242. Kainulainen V, Korhonen TK. Dancing to another tune-adhesive moonlighting proteins in bacteria. Biology (Basel) 2014; 3(1):178–204.

243. Antikainen J, Anton L, Sillanpää J, Korhonen TK. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Mol. Microbiol. 2002; 46(2):381–394.

244. Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, Muscariello L, Marasco R, Sacco M. Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. Microb. Cell Fact. 2009; 8:14.

245. Rodrigues KL, Caputo LR, Carvalho JC, Evangelista J, Schneedorf JM. Antimicrobial and healing activity of kefir and kefiran extract. Int. J. Antimicrob. Agents. 2005; 25(5):404–408.

246. Huseini HF, Rahimzadeh G, Fazeli MR, Mehrazma M, Salehi M. Evaluation of wound healing activities of kefir products. Burns. 2012; 38(5):719–723.

247. Hessle C, Andersson B, Wold AE. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect. Immun. 2000; 68(6):3581–3586.

248. Jensen JM, Förl M, Winoto-Morbach S, Seite S, Schunck M, Proksch E, Schütze S. Acid and neutral sphingomyelinase, ceramide synthase, and acid ceramidase activities in cutaneous aging. Exp. Dermatol. 2005; 14(8):609–618.

249. Mizutani Y, Mitsutake S, Tsuji K, Kihara A, Igarashi Y. Ceramide biosynthesis in keratinocyte and its role in skin function. Biochimie. 2009; 91(6):784–790.

250. Sugimoto S, Iwamoto T, Takada K, Okuda K, Tajima A, Iwase T, Mizunoe Y. *Staphylococcus epidermidis* Esp degrades specific proteins associated with *Staphylococcus aureus* biofilm formation and host-pathogen interaction. J. Bacteriol. 2013; 195(8):1645–1655.

251. Hacini-Rachinel F, Gheit H, Le Luduec JB, Dif F, Nancey S, Kaiserlian D. Oral probiotic control skin inflammation by acting on both effector and regulatory T cells. PLoS One. 2009; 4(3):e4903.

252. Iemoli E, Trabattoni D, Parisotto S, Borgonovo L, Toscano M, Rizzardini G, Clerici M, Ricci E, Fusi A, De Vecchi E, Piconi S, Drago L. Probiotics reduce gut microbial translocation and improve adult atopic dermatitis. J. Clin. Gastroenterol. 2012; (S46):S33–S40.

253. Al-Ghazzewi FH, Tester RF. Effect of konjac glucomannan hydrolysates and probiotics on the growth of the skin bacterium *Propionibacterium acnes in vitro*. Int. J. Cosmet. Sci. 2010; 32(2):139–142.

254. Mayes T, Gottschlich MM, James LE, Allgeier C, Weitz J, Kagan RJ. Clinical safety and efficacy of probiotic administration following burn injury. J. Burn Care Res. 2015; 36(1):92–99.

255. Shah KR, Boland CR, Patel M, Thrash B, Menter A. Cutaneous manifestations of gastrointestinal disease: part I. J. Am. Acad. Dermatol. 2013; 68(2):189.e1–21.

256. Guillemard E, Tondu F, Lacoin F, Schrezenmeir J. Consumption of a fermented dairy product containing the probiotic *Lactobacillus casei* DN-114001 reduces the duration of respiratory infections in the elderly in a randomised controlled trial. Br. J. Nutr. 2010; 103(1):58–68.

258. Varian BJ, Poutahidis T, DiBenedictis BT, Levkovich T, Ibrahim Y, Didyk E, Shikhman L, Cheung HK, Hardas A, Ricciardi CE, Kolandaivelu K, Veenema AH, Alm EJ, Erdman SE. Microbial lysate upregulates host oxytocin. Brain Behav. Immun. 2017; 61:36–49.

258. Erdman SE, Poutahidis T. Probiotic 'glow of health': It's more than skin deep. Benef. Microbes. 2014; 5(2):109–119.

259. Poutahidis T, Kearney SM, Levkovich T, Qi P, Varian BJ, Lakritz JR, Ibrahim YM, Chatzigiagkos A, Alm EJ, Erdman SE. Microbial symbionts accelerate wound healing via the neuropeptide hormone oxytocin. PLoS One. 2013; 8(10):e78898.

260. Levkovich T, Poutahidis T, Smillie C, Varian BJ, Ibrahim YM, Lakritz JR, Alm EJ, Erdman SE. Probiotic bacteria induce a 'glow of health' PLoS One. 2013; 8(1):e53867.

261. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat. Rev. Immunol. 2003; 3(4):331–341.

262. Kaplan B, Gönül B, Dinçer S, Dinçer Kaya FN, Babül A. Relationships between tensile strength, ascorbic acid, hydroxyproline, and zinc levels of rabbit full-thickness incision wound healing. Surg. Today. 2004; 34(9):747–751.

263. Rutherfurd SM, Chung TK, Thomas DV, Zou ML, Moughan PJ. Effect of a novel phytase on growth performance, apparent metabolizable energy, and the availability of minerals and amino acids in a low-phosphorus corn-soybean meal diet for broilers. Poult. Sci. 2012; 91(5):1118–1127.

264. Askelson TE, Campasino A, Lee JT, Duong T. Evaluation of phytate-degrading *Lactobacillus* culture administration to broiler chickens. Appl. Environ. Microbiol. 2014; 80(3):943–950.

265. Rooj AK, Kimura Y, Buddington RK. Metabolites produced by probiotic lactobacilli rapidly increase glucose uptake by Caco-2 cells. BMC Microbiol. 2010; 10:16.

266. De Angelis M, Bottacini F, Fosso B, Kelleher P, Calasso M, Di Cagno R, Ventura M, Picardi E, van Sinderen D, Gobbetti M. *Lactobacillus rossiae*, a vitamin B12 producer, represents a metabolically versatile species within the genus *Lactobacillus*. PLoS One. 2014; 9(9):e107232.

267. Oda Y, Tu CL, Menendez A, Nguyen T, Bikle DD. Vitamin D and calcium regulation of epidermal wound healing. J. Steroid Biochem. Mol. Biol. 2016; 164:379–385.

268. Jones ML, Martoni CJ, Prakash S. Oral supplementation with probiotic *L. reuteri* NCIMB 30242 increases mean circulating 25-hydroxyvitamin D: a *post hoc* analysis of a randomized controlled trial. J. Clin. Endocrinol. Metab. 2013; 98(7):2944–2951.

269. Roager HM, Sulek K, Skov K, Frandsen HL, Smedsgaard J, Wilcks A, Skov TH, Villas-Boas SG, Licht TR. *Lactobacillus acidophilus* NCFM affects vitamin E acetate metabolism and intestinal bile acid signature in monocolonized mice. Gut Microbes. 2014; 5(3):296–303.

270. Degirolamo C, Rainaldi S, Bovenga F, Murzilli S, Moschetta A. Microbiota modification with probiotics induces hepatic bile acid synthesis via downregulation of the Fxr-Fgf15 axis in mice. Cell Rep. 2014; 7(1):12–8.

271. Arck P, Handjiski B, Hagen E, Pincus M, Bruenahl C, Bienenstock J, Paus R. Is there a 'gut-brain-skin axis'? Exp. Dermatol. 2010; 19(5):401–405.

272. Holzer P, Farzi A. Neuropeptides and the microbiota-gut-brain axis. Adv. Exp. Med. Biol. 2014; 817:195–219.

273. Deing V, Roggenkamp D, Kühnl J, Gruschka A, Stäb F, Wenck H, Bürkle A, Neufang G. Oxytocin modulates proliferation and stress responses of human skin cells: implications for atopic dermatitis. Exp. Dermatol. 2013; 22(6):399–405.

274. Ndiaye K, Poole DH, Pate JL. Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. Biol. Reprod. 2008; 78(4):786–793.

275. Macciò A, Madeddu C, Chessa P, Panzone F, Lissoni P, Mantovani G. Oxytocin both increases proliferative response of peripheral blood lymphomonocytes to phytohemagglutinin and reverses immunosuppressive estrogen activity. *In Vivo*. 2010; 24(2):157–163.

276. Li H, Qiu T, Huang G, Cao Y. Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. Microb. Cell Fact. 2010; 9:85.

277. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, Koga Y, Sudo N. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am. J. Physiol. Gastrointest. Liver Physiol. 2012; 303(11):G1288–G1295.

278. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, Bisson JF, Rougeot C, Pichelin M, Cazaubiel M, Cazaubiel JM. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br. J. Nutr. 2011; 105(5):755–764.

279. de Almeida TF, de Castro Pires T, Monte-Alto-Costa A. Blockade of glucocorticoid receptors improves cutaneous wound healing in stressed mice. Exp. Biol. Med. (Maywood). 2016; 241(4):353–358.

280. Abd El-Aleem SA, Abdelwahab S, Am-Sherief H, Sayed A. Cellular and physiological upregulation of inducible nitric oxide synthase, arginase, and inducible cyclooxygenase in wound healing. J. Cell. Physiol. 2019; 234(12):23618-23632.

281. Broughton G, Janis JE, Attinger CE. Wound healing: An overview. Plast. Reconstr. Surg. 2006; 117(S7):1e-S-32e-S

282. Frank S, Kampfer H, Wetzler C Pfeilschifter J. Nitric oxide drives skin repair: Novel functions of an established mediator. Kidney Int. 2002; 61(3):882-888

283. Kumar S, Singh RK, Bhardwaj TR. Therapeutic role of nitric oxide as emerging molecule. Biomed. Pharmacother. 2017; 85:182–201.

284. Bernatchez SF, Menon V, Sto el J, Walters SA, Lindroos WE, Crossland MC, Shawler LG, Crossland SP, Boykin JV Jr. Nitric oxide levels in wound fluid may reflect the healing trajectory. Wound Repair Regen. 2013; 21(3):410-417

285. Shekhter AB, Serezhenkov VA, Rudenko TG, Pekshev AV, Vanin AF. Beneficial effect of gaseous nitric oxide on the healing of skin wounds. Nitric Oxide. 2005; 12(4):210–219.

286. Yamasaki K, Edington H.D, McClosky C, Tzeng E, Lizonova A, Kovesdi I, Steed D.L, Billiar TR. Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. J. Clin. Investig. 1998; 101(5):967–971.

287. Shi HP, Efron DT, Most D, Tantry US, Barbul A. Supplemental dietary arginine enhances wound healing in normal but not inducible nitric oxide synthase knockout mice. Surgery. 2000; 128(2):374–378.

288. Malone-Povolny MJ, Maloney SE, Schoenfisch MH. Nitric oxide therapy for diabetic wound healing. Adv. Healthc. Mater. 2019; 8(12):e1801210.

289. Amadeu TP, Costa AM. Nitric oxide synthesis inhibition alters rat cutaneous wound healing. J. Cutan. Pathol. 2006; 33(7):465–473.

290. Frank S, Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J. Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair. FASEB J. 1999; 13:2002–2014.

291. Kitano T, Yamada H, Kida M, Okada Y, Saika S, Yoshida M. Impaired healing of a cutaneous wound in an inducible nitric oxide synthase-knockout mouse. Dermatol. Res. Pract. 2017; 2017:2184040.

292. Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J, Frank S. The function of nitric oxide in wound repair: Inhibition of inducible nitric oxide-synthase severely impairs wound reepithelialization. J. Investig. Dermatol. 1999; 113(6):1090–1098.

293. Cinque B, La Torre C, Lombardi F, Palumbo P, Van der Rest M, Cifone M.G. Production conditions affect the *in vitro* antitumoral effects of a high concentration multi-strain probiotic preparation. PLoS One. 2016; 11(9):e0163216.

294. Cinque B, La Torre C, Lombardi F, Palumbo P, Evtoski Z, Jr Santini S, Falone S, Cimini A, Amicarelli F, Cifone MG. VSL#3 probiotic differently influences IEC-6 intestinal epithelial cell status and function. J. Cell. Physiol. 2017; 232(12):3530-3539.

295. Biagioli M, Laghi L, Carino A, Cipriani S, Distrutti E, Marchianò S, Parolin C, Scarpelli P, Vitali B, Fiorucci S. Metabolic variability of a multispecies probiotic preparation impacts on the anti-inflammatory activity. Front. Pharmacol. 2017; 8:505.

296. Trinchieri V, Laghi L, Vitali B. Efficacy and Safety of a Multistrain probiotic formulation depends from manufacturing. Front. Immunol. 2017; 8:1474.

297. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell. Biol. Toxicol. 2005; 21(1):1-26.

298. Varasteh S, Fink-Gremmels J, Garssen J, Braber S. α-Lipoic acid prevents the intestinal epithelial monolayer damage under heat stress conditions: model experiments in Caco-2 cells. Eur. J. Nutr. 2018; 57(4):1577-1589.

299. Zhang J, Lu Y, Wei J, Li L, Han L. Protective effect of carboxytmethylpachymaran on TNF-α-induced damage in Caco-2 cell monolayers. Int. J. Biol. Macromol. 2016; 93(Pt A):506-511.

300. Anderson RC, Cookson AL, McNabb WC, Kelly WJ, Roy NC. *Lactobacillus plantarum* DSM 2648 is a potential probiotic that enhances intestinal barrier function. FEMS Microbiol. Lett. 2010; 309(2):184-192.

301. Morini J, Babini G, Barbieri S, Baiocco G, Ottolenghi A. The interplay between radioresistant Caco-2 cells and the immune system increases epithelial layer permeability and alters signaling protein spectrum. Front. Immunol. 2017; 8:223.

302. Geback T, Schulz MM, Koumoutsakos P, Detmar M. TScratch: A novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques. 2009; 46(4):265–274.

303. La Torre C, Cinque B, Lombardi F, Miconi G, Palumbo P, Evtoski Z, Placidi G, Fanini D, Cimini AM, Benedetti E, Giuliani M, Cifone MG. Nitric oxide chemical donor affects the early phases of *in vitro* wound healing process. J. Cell. Physiol. 2016; 231(10):2185–2195.

304. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: Mechanisms, signaling, and translation. Sci. Transl. Med. 2014; 6(265):265sr6.

305. Yu Y, Dunaway S, Champer J, Kim J, Alikhan A. Changing our microbiome: Probiotics in dermatology. Br. J. Dermatol. 2020; 182(1):39-46.

306. Notay M, Foolad N, Vaughn AR, Sivamani RK. Probiotics, prebiotics, and synbiotics for the treatment and prevention of adult dermatological diseases. Am. J. Clin. Dermatol. 2017; 18(6):721-732.

307. Tsiouris CG, Kelesi M, Vasilopoulos G, Kalemikerakis I, Papageorgiou EG. The efficacy of probiotics as pharmacological treatment of cutaneous wounds: Meta-analysis of animal studies. Eur. J. Pharm. Sci. 2017; 104:230–239.

308. Kumar M, Kissoon-Singh V, Coria AL, Moreau F, Chadee K. Probiotic mixture VSL#3 reduces colonic inflammation and improves intestinal barrier function in Muc2 mucin-deficient mice. Am. J. Physiol. Gastrointest. Liver Physiol. 2017; 312(1):G34-G45.

309. Primavera R, Palumbo P, Celia C, Cinque B, Carata E, Carafa M, Paolino D, Cifone MG, Di Marzio L. An insight of *in vitro* transport of PEGylated non-ionic surfactant vesicles (NSVs) across the intestinal polarized enterocyte monolayers. Eur. J. Pharm. Biopharm. 2018; 127:432-442.

310. Xiao G, Tang L, Yuan F. Eicosapentaenoic acid enhances heat stress-impaired intestinal epithelial barrier function in Caco-2 cells. PLoS One. 2013; 8(9):e73571.

311. Grześkowiak L, Isolauri E, Salminen S, Gueimonde M. Manufacturing process influences properties of probiotic bacteria. Br. J. Nutr. 2010; 105(6):887-894.

312. Clements ML, Levine MM, Ristaino PA, Daya VE, Hughes TP. Exogenous lactobacilli fed to man - their fate and ability to prevent diarrheal disease. Prog. Food Nutr. Sci. 1983; 7(3-4):29–37.

313. Lipinska L, Klewicki R, Elżbieta K, Kolodziejczyk K, Sójka M, Nowak A. Antifungal activity of *Lactobacillus* sp. bacteria in the presence of xylitol and galactosyl-xylitol. Biomed. Res. Int. 2016; 2016:5897486.

314. Ott JM, Podolsky DK. Functional modulation of enterocytes by grampositive and gram-negative microorganisms. Am. J. Physiol. Gastrointest. Liver Physiol. 2004; 286(4):G613- G626.

315. Krishnan M, Penrose HM, Shah NN, Marchelletta RR, McCole DF. VSL#3 probiotic stimulates T-cell protein tyrosine phosphatase–mediated recovery of IFN-γ–induced intestinal epithelial barrier defects. Inflamm. Bowel Dis. 2016; 22(12):2811-2823.

316. Shibolet O, Karmeli F, Eliakim R, Swennen E, Brigidi P, Gionchetti P, Campier M, Morgenstern S, Rachmilewitz D. Variable response to probiotics in two models of experimental colitis in rats. Inflamm. Bowel Dis. 2002; 8(6):399-406.

317. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J. Immunol. 2005; 174(6):3237-3246.

318. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, Senninger N, Bruewer M. Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 2009; 296(5):G1140- G1149.

319. Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp. Mol. Med. 2018; 50(8):103.

320. Allain T, Amat CB, Motta JP, Manko A, Buret AG. Interactions of Giardia sp. with the intestinal barrier: Epithelium, mucus, and microbiota. Tissue Barriers. 2017; 5(1):e1274354.

321. Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: A double-blind, placebo-controlled trial. Gastroenterology, 2000; 119(2):305-309.

322. Halpern MD, Denning PW. The role of intestinal epithelial barrier function in the development of NEC. Tissue Barriers. 2015; 3(1-2):e1000707.

323. Moore SA, Nighot P, Reyes C, Rawat M, McKee J, Lemon D, Hanson J, Ma TY. Intestinal barrier dysfunction in human necrotizing enterocolitis. J. Pediatr. Surg. 2016; 51(12):1907-1913.

324. Grześkowiak Ł, Isolauri E, Salminen S, Gueimonde M. Manufacturing process influences properties of probiotic bacteria. Br. J. Nutr. 2010; 105:887-894.

325. Zacarias MF, Souza TC, Zaburlin N. Influence of technological treatments on the functionality of *bifidobacterium lactis* INL1, a breast milk-derived probiotic. J. Food Sci., 2017, 82(10):2462-2470.

326. Fenster K, Freeburg B, Hollard C, Wong C, Rønhave Laursen R, Ouwehand AC. The Production and Delivery of Probiotics: A Review of a Practical Approach. Microorganisms. 2019, 7(3). pii: E83.