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Probiotical Cell Fragments (PCFs) as "Novel Nutraceutical Ingredients"

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Abstract

Probiotical cell fragments (PCFs) are structural components of the probiotic cell lysate(s) and exhibit similar beneficial effects on the host as live probiotic bacteria. With cell fragment technology (CFT™), the structural fragments are isolated and purified from live probiotic cells. While observed to be strain-dependent as in the case of live probiotics, orally administered PCFs demonstrated a broad spectrum of immune modulation functions; anti-allergy; anti-inflammation; anti-bacterial and anti-viral properties; anti-mutagenic; and radioprotective and detoxification abilities in humans and animals. The PCFs mechanisms of action include events of motifs of cell wall peptidoglycans (PGs), DNA motifs, nucleotide containing components, lipoteichoic acids (LTAs), surface layer (S-layer) proteins, and cellular carbohydrates. Different immunological *in vivo-in vitro* tests have shown that PCFs, essentially, have the ability to stimulate the macrophages, and induce cytokines such as interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), and natural killer (NK) cells. PCFs may be used as ingredients for foods and beverages or as nutritional supplements with long term stability and shelf-life up to 5 years. PCFs may also be used as health restorative ingredients in cosmetic products. The outcome of probiotics CFT™ stands as an advantage to the food and pharmaceutical industries, regarding the formulation of unique products with unadulterated sensory characteristics of origin. Hence, PCFs are being characterized here as "novel nutraceutical ingredients" for health maintenance in both humans and animals.

Keywords

Probiotical Cell Fragments (PCFs), Paraprobiotics, Immunobiotics, Immunomodulator, Nutraceutical Ingredients, Lactic Acid Bacteria (LAB)

1. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [1]. In contrast, paraprobiotics are defined as non-viable microbial cells (intact or broken) or crude cell extracts which when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal

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consumer [2]. The prefix "para" has been preferred for its interpretation of "alongside of" or "atypical", which can simultaneously display similarity to and difference from the traditional probiotic definition. When isolated and purified, the dynamic cellular structures from probiotics or paraprobiotics (including their derivatives or metabolites) can be categorized into different fragments. These active fragments, metabolites, or derivatives are expressed as immunogenics (**Figure 1**) [3]-[5]. Immunogenics include intracellular and extracellular bacterial components with immunoregulatory abilities, such as peptidoglycan (PG), lipoteichoic acid (LTA), extracellular phosphopolysaccharides, and DNA/RNA [4]. Paraprobiotics in particular, contain many structural fragments with physiological functions, such as cell wall PGs, teichoic acids (TAs), nucleotide containing components of DNA and/or RNA motifs, surface layer (S-layer) proteins, secreted proteins, and various cell wall-associated polysaccharides (CPSs) as shown in **Figure 2**. Among these fragments of which some are immunogenics, the expression "probiotical cell fragments" or PCFs is assigned to them (see **Figure 3**).

2. Background of PCFs as the Potent Immune Response Modifiers

By the use of cell fragment technology (CFT™), the structural fragments can be isolated and purified from the renowned live probiotic cells. For example, cell wall of lactobacilli contains several distinctive structural elements, consisting of the multilayered PG sacculus, namely, muramyl-peptides (MPs), muramyl dipeptides (MDPs), and muramyl polypeptides (MPPs), which are seeded with TAs, cellular polysaccharides, and some other proteins. TAs are the second main constituents of the cell walls and usually contain repeating units of polyribitol phosphate or polyglycerol phosphate (Glc) covalently anchored to PG (see **Figure 2**). The TAs hold

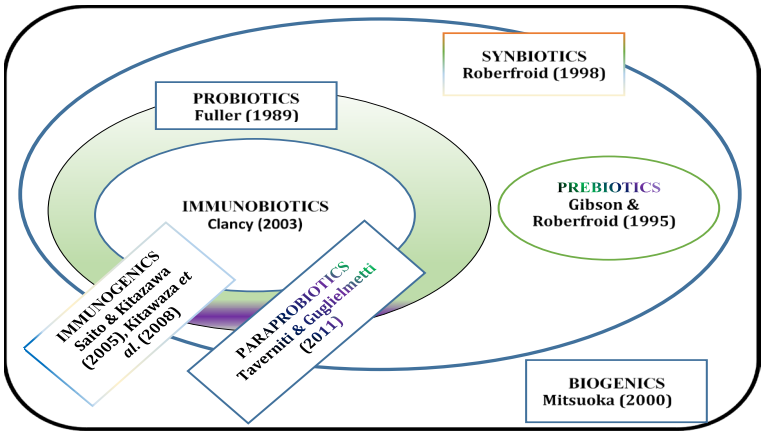

Figure 1. Diagram representation of the interactions between the modern concepts of probiotic and related terms (Modified from reference [5].

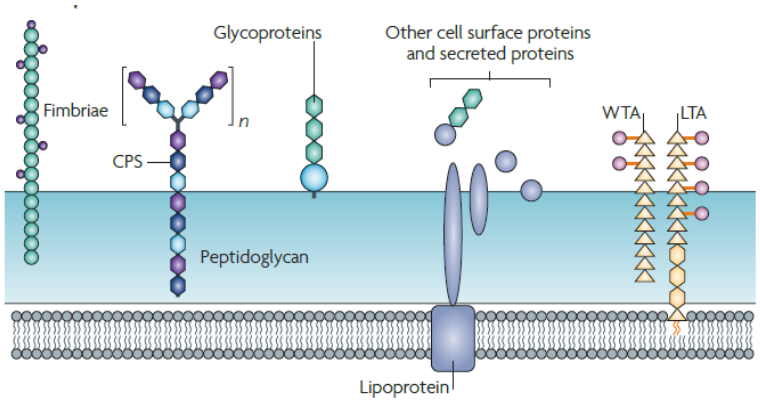

Figure 2. Example of isolated PCFs within a structure of gram (+) paraprobiotics (Adapted from reference [6]).

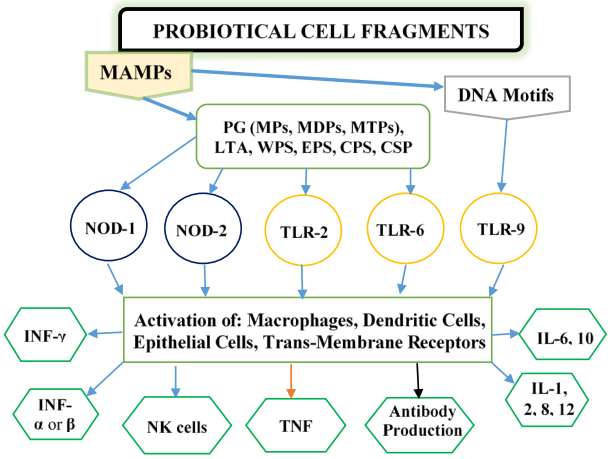

Figure 3. Immunoactivation mechanisms of immunogenic PCFs; PG is peptidoglycan, MPs are muramyl peptides; MDPs are muramyl dipeptides; MTPs are muramyl tripeptides; LTA is lipoteichoic acid; WPS is wall polysaccharides; EPS is exopolysaccharides; CPS is capsular polysaccharides, and CSP is cellular surface proteins.

2 types of polymers: wall-teichoic acids (WTAs) and lipoteichoic acids (LTAs) [7]. Interestingly, only LTAs are immunogenics, which have been identified as a key factor for triggering the synergistic induction of interleukin (IL)-10 production [8]. The fragments of genome immunogenic DNA motifs have been found to induce immunoglobulin secretion and B-cell proliferation, or inhibited IL-8 secretion by HT-29 cells stimulated with tumor necrosis factor-α (TNF-α) [5].

In addition, genome DNA motifs from immunobiotic lactic acid bacteria (LAB) induce the immunoactivation of gut-associated lymphoid tissue (GALT). The term immunobiotics refers to probiotic strains that are beneficially able to regulate the mucosal immune system [9]. It has been also established that the nucleotide-binding oligomerization domain-containing protein-1 (NOD-1), NOD-2, toll-like receptor-2 (TLR-2), and TLR-9 are able to identify or recognize the DNA motifs and cell wall components of dietary LAB, thereby contributing to immunoregulation in the GALT [3]. With respect to immunoactivation mechanisms caused by immunogenic PCFs, mechanisms mediated by different TLRs and NOD-like receptors (NLRs) as pattern recognition receptors (PRRs) have recently been uncovered, and found to be part of the complex innate immune system in both humans and animals (see **Figure 3**).

The polysaccharides within paraprobiotic cell wall possess 3 types: PG glycan strand (known as wall polysaccharides or WPS), exopolysaccharides (EPS), and capsular polysaccharides or CPS [7]. Among them, only WPS and EPS are immunogenics. Overall, MPs, MDPs, MTPs, LTAs, WPS, EPS, and some surface proteins are immunogenics which have been clinically proven to interact with different PRRs from intestinal epithelial cells (IECs) and mucosal immune cells, thereby modulating the mucosal immune system. It is necessary to emphasize that there are 3 classes of PRRs which spot and suspect viral components: 1) retinoic acid-inducible gene I like receptors (RLRs), 2) TLRs, and 3) NLRs [5]. Remarkably, carbohydrates and glycolipids (which exist in many microbes) are also known to activate epithelial cells through interaction with TLRs [10].

3. PCFs as MAMPs and PRRs as Host Receptors

As delineated in **Figure 3**, the PCFs may serve as microbe-associated molecular patterns (MAMPs) because they activate the corresponding PRRs of the innate immunity. The PRRs recognize the PCFs as MAMPs, which are naturally popular and conserved among many probiotics, often being located on the bacterial surface or secreted molecules [11]. Thus far, several MAMPs of immunobiotics that can be connected to the famous host-responses have been identified [6], and in many cases, these effector molecules are associated with the bacterial cell wall [7]. The most notable PRRs include TLRs, the NLRs, the RIG-I-like RNA helicases, the C-type lectin receptors, and cytosolic DNA sensors [5]. These receptors brilliantly fine tune signals that specifically recognize

the quantity and quality of conserved moieties associated with MAMPs including carbohydrates, lipids, proteins, lipoproteins, bacteriocins, and nucleic acids. Following binding of the moieties to PRRs, activation of the innate immune system leads to diverse cellular responses including the induction of interferon regulatory factors (IRFs), activator protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) which all regulate the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and type I interferon (IFN) [12].

TLRs are trans-membrane proteins present at the cell surface or on the membrane of endocytic vesicles of both IECs and intestinal dendritic cells (DCs). Interestingly, PCFs encounter both intestinal DCs and IECs which are key players in both innate and adaptive immunity by means of their PRRs. In addition to TLRs, the NLRs also recognize MAMPs and are known to transmit signals on the communication with PCFs (see NOD-1 and NOD-2 in **Figure 3**). The PCFs are, therefore, be able to modulate the production of cytokines and regulate the immune system. Besides, the PCFs also activate the corresponding receptors of adaptive immunity towards allergy tolerance [13] (see **Figure 4**).

With regards to immunostimulatory DNA motifs, it has been reported that cytosine-guanosine dinucleotides (CpG)-DNA derived from bacteria stimulates B lymphocytes [5] and lead to a huge surge of interest in immunostimulatory DNA. As part of the PCFs, immunostimulatory DNA motifs induce interferon IFN-α and IFN-γ and are also strong activator of natural killer (NK) cells. The TLR-9 has been long recognized as a receptor molecule for CpG-DNAs, [14] and as a result, our understanding of the effect of DNA motifs within the PCFs on the immune system has also advanced through the TLR-9 (see **Figure 3**). The TLRs have been considered to be ideal for natural defense mechanisms, and for each TLR, there are unique identification molecules, which are bacterial modulins [15] [16]. In general, the TLRs are able to recognize bacterial modulins having MAMPs or pathogen-associated molecular patterns (PAMPs) and exert the cytokine-mediated regulation over the immune response [17].

4. History of Safe Use of PCFs

The PCFs are borne of a half-century research by the European scientists [18], and their value to new and innovative probiotic lysates has a shared long history of more than a decade in Russia, Europe, Japan, and United States of America. Practically, all the strains used in CFT™ are considered commensal microbiota, with no pathogenic potential, and most have passed the evaluation of the European Union (EU) novel food regulations. LAB and the yeast *Saccharomyces* are the well-known probiotics. Strains of the genera *Lactobacillus* and *Bifidobacterium* are the utmost quantified with safety, and a number of them have gained a generally recognized as

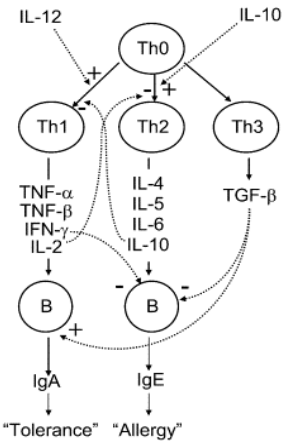

Figure 4. Development of the "allergic" [Type 2 T helper cells (Th2)] or "tolerant" (Th1) phenotype. IL-12 stimulates a shift toward the tolerant phenotype, whereas IL-10 stimulates the provision of the allergic phenotype. Th3 cells, through the production of transforming growth factor-β (TGF- β), further fuel the trend toward tolerance. On the other hand, Immunoglobulin A (IgA) supports allergy elimination; IgE may activates mast cells and cause allergic symptoms. Adapted from reference [13].

safe (GRAS) status. Other genera of *Streptococcus*, *Bacillus* or *Enterococcus* cover numerous opportunistic pathogens. Clinical and experimental analyses have drawn scrutiny to many cases of prevention and treatment of gastrointestinal (GI) disorders, allergies, colonic cancer, wound healings, sinus, flu, and other acute and chronic infections/diseases or daily discomforts. A majority of these could be expressively resolved safely with the use of paraprobiotics or PCFs. Most of the PCF formulations have been tested and found to be effective in the prevention and treatment of all the previous stated concerns, including the stomach colonization by pathogens in humans, immune deficiency, as well as infectious lesions of bacterial, viral and fungal etiology [18]. The mechanisms of action for PCFs on some of the essential health conditions are described separately below. Overall, the established PCFs are considered as highly safe.

5. Gastrointestinal Digestion and Absorption

It is necessary to point out that the GI ecosystem features dynamic and reciprocal interactions among its commensal microbiota, the epithelium, and the immune system. As probiotics and/or paraprobiotics are part of the gut flora (either naturally or by supplementation), an understanding of the mechanisms whereby PCFs compel their immune modulating effects on the host requires an outline of what position PCFs occupy in the gut flora. It is also required to understand how the commensal microbiota within the stomach interact and/or support the immune system of the own host. Moreover, systemic induction of minor inflammations is believed to be considerable in PCF effects against atopic eczema-dermatitis syndrome. Similarly, application for PCFs aimed at enhancing the host immune response, such as those used for the prevention of gastroenteritis or traveler's diarrhea, also seem to require special PCF formulations with a slight immunostimulatory activity. In contrast, for inflammatory bowel disease (IBD) treatment, the PCFs that can correct the pro-inflammatory pathology may be formulated a bit differently, taking the damaged epithelial barrier into account. The combinations of both viable probiotic cells and non-viable PCFs are preferred in this case. It is remarkably clear in this article that PCFs may be used as "novel nutraceutical ingredients" with the intentions of promoting excellent health, preventing and treating of infectious diseases, anti-allergies, anti-tumor or anti-colon cancer, anti-inflammatory diseases, wounds healing, and many more. Along these lines, PCFs look to establish a consensus in the development of novel nutraceutical products that are of beneficial to food and pharmaceutical industries.

5.1. PCFs as a Source of Essential and Nonessential Amino Acids

The PCFs represent a source not only of muramyl peptides with biological activities but also their derived vital amino acids and organic nitrogen. The MPs, MDPs, and MTPs with biological activities (shown in **Figure 3**) are of significant importance as they are well characterized within PG of paraprobiotical cell wall. It has been reported that the stem peptides in those muramyl peptides contain 4 alternating D- and L-amino acids with biological activities [19]. The 1st amino acid of the MTPs is L-alanine, the 2nd and 4th amino acids are D-glutamine and D-alanine, respectively. L-lysine is the 3rd amino acid in most Gram (+) bacteria, whereas, in some Gram (+) bacilli and the majority of Gram (–) bacteria, it is *meso*-diaminopimelic acid (*meso*-DAP) [19]. The consensus sequence of the amino acids in lactobacilli is L-Ala/D-Glu/(L-Lys or *meso*-DAP)/D-Ala/D-Ala [6]. Moreover, D-asparagine is often used as a cross-bridge between L-lysine and D-alanine and the rest may also be amidated [6]. It is necessary to acknowledge that a depletion of L-arginine has been implicated in the pathogenesis. Apparently, the use of L-Arg by trophozoites dramatically reduces the availability of this amino acid to synthesize NO, a compound that inhibits growth, encystation and excystation of *Giardia* [20]. Besides, low luminal concentration of L-Arg has been reported to reduce enterocyte movements, thus providing the parasite with a more stable environment for colonization [20] [21].

5.2. Mechanisms of Action

As "novel nutraceutical ingredients," PCFs are equally subjected to both digestion and absorption processes in the GI tract. They can first interact within the intestinal tract and subsequently exert their targeted needful activities in various peripheral organs subsequent to absorption. For the PCFs to become bioactive, they must be provided in sufficient amount during GI digestion and then regulated before and after absorption. It means that GI processes regulate the supply of digested PCFs including their amino acids and/or nitrogen into the body, and consequently, their metabolic utilization. As indicated earlier on, the key PCFs include the PGs, TAs, WPS, EPS,

CPS, and different surface proteins. PG has 3 distinguished peptides: MPs, MDPs, and MTPs. These 3 PG fragments, for example, must first be hydrolyzed by lysozyme or remodeled through autolysis action in the gut before they could be detected by PRRs [6]. In addition to that, the PGs are ligands for TLR-2, with CD14 as a co-receptor [6]. On the other hand, the principal components of TAs are WTAs and LTAs. Naturally, the first digestion stage of TAs and other cellular surface proteins or secreted proteins entails pepsin hydrolysis and acid secretion in the stomach. Moreover, most of the digested PCFs are expected to be readily absorbable in an intact form as soon as they are emptied into the duodenum. However, some of the fragments are expected to precipitate in the stomach and then gradually released into the small intestine in the form of degraded fragments. In the duodenum, these cell fragments would be further subjected to pancreatic enzymes.

To our knowledge, the PCFs have been found in the intestinal lumen (in intact and/or degraded formats), whereby they activated the TLRs, leading to a proliferation-inducing ligand (APRIL) and the production of B cell activating factor (BAFF) [22]. These 2 cytokines are also known for promoting all kinds of IgA class-switching responses in the intestines [22]. As the intestinal tail contains numerous peptidases such as pepsin, elastase, trypsin, chymotrypsin, and carboxypeptidase, the PGs are expected to be degraded into MTPs, MDPs, MPs, other smaller peptides, and amino acids, which would be eventually absorbed. Absorption of the digested PCFs from the intestine is anticipated to be specific too. In contrast, some PGs may be relatively resistant to the proteolytic digestive enzymes and thus, may also reach their core targets intactly. To be specific, proline or lysine substance containing MPs are likely to be highly resistant to proteolytic degradation. MDPs, on the other hand, are reported to have been taken up by the intestinal peptide transporter PEPT1 (known as SLC15A1) in intact form [6]. In comparison to the mechanisms of action outlined in **Figure 3**, other research also described that the acid-digested CPS induces production of higher IFN-γ and lower IL-4 in mice splenocytes than in water treated mice [23]. This discovery confirms the role of gastric acid in providing a variety of different PCFs as nutrients that are made readily available to the body for absorption. That is to say; the digested PCFs can be absorbed by Peyer's patches through microfold (M) cells in the intestinal tract and modulate antigen-presenting cells (APCs) such as DCs, through the TLRs or NLRs. That may result in a selective enhancement of Th1 cell proliferation, and the subsequent production of IFN-γ and IL-2 (see **Figure 4**), which are dynamic and powerful cytokines for cell-mediated immune responses [23]. The point is that, IFN-γ does not only activates macrophages and NK cells, but also selectively inhibits the proliferation of Th2 cells, which produce cytokines such as IL-4 and a few others (see **Figure 4**). Interestingly, an apical intestine alkaline phosphatase (IAP) enzyme has been found to reduce lipopolysaccharide (LPS)-TLR-4 signaling by detoxifying LPS [6].

Most of the PCFs including the bioactive MPs may operate directly on their spectacular targets existing in the GI tract. The MDPs and MTPs may also cross the intestine and reach the peripheral target sites. Apart from intestinal peptide transporter PEPT1 or as branded as SLC15A1, other specific transport arrangements, such as trans-cytosis (which is implicated in macromolecules and microorganisms transport by M cells) [19] and endocytosis (of the viral envelope proteins, DNA and RNA, outside of cells and in cytoplasmic vacuoles after phagocytosis) have been described [24]. The absorption and transportation of digested PCFs into the Peyer's patches through the M cells would be processed further by the professional APCs (DCs or macrophages) before entering the mesenteric lymph nodes. Alternatively, luminal PCFs may enter lamina propria either via M cells (primarily particulate antigen) overlying the Peyer's patch through the enterocytes (soluble antigen), or potentially via capture by traversing DCs as per interpretation of reference [25]. The mesenteric lymph nodes and Peyer's patches are believed to be the 2 main inductive sites (see **Figure 5**). The Peyer's patches are situated mainly on the antimesenteric side of the small intestine and include a number of B cell rich zones with germinal centers called follicles. So adjacent to these in the parafollicular areas, there are T cell rich zones [25]. After intracellularly processing and handling of the digested PCFs, the immune cells travel from lamina propria and Peyer's patches to mesenteric lymph nodes from where they can enter the bloodstream via the thoracic duct (see **Figure 5**).

However, during PCFs processing and through interaction within immunocompetent cells, most of the PCFs are expected to exert the immunomodulatory effects by inducing the right cytokine production of immunocompetent cells. In essence, this appears to demonstrate how the PCFs may be useful in preventing cytokine mediated GI and/or respiratory diseases. Cytokines are known by their influence to stimulate or suppress cell functions [25]. Furthermore, degraded PCFs can also be found in the blood in degraded format. The enzymatic proteolysis may produce other bioactive MPs or simple peptides in the body too. However, it should be noted that the specific MPs, MDPs, MTPs, or other peptides may be formed by proteolytic bacterial species, and they are likely to be less effective in their original format if not prepared intra-cellularly.

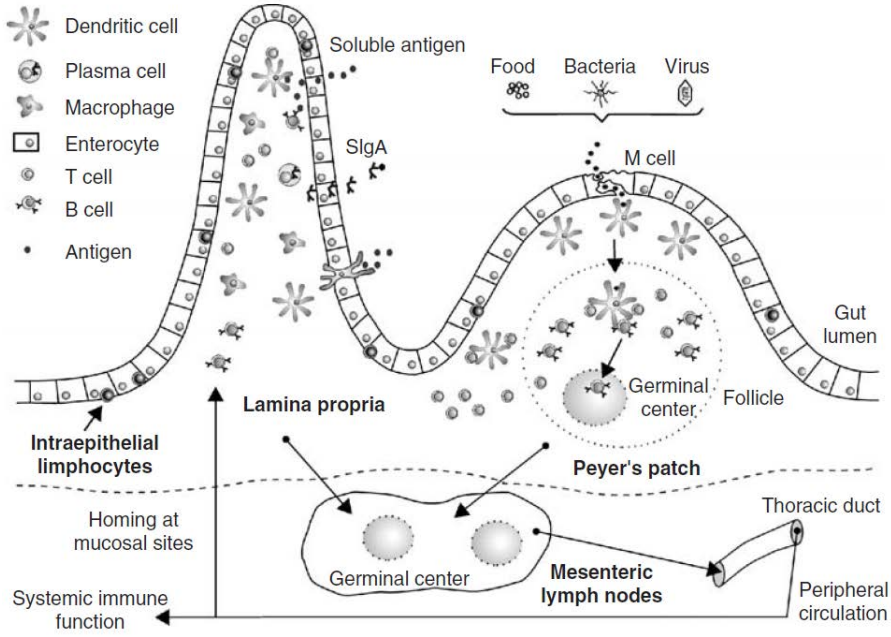

Figure 5. Schematic representation of the gut-associated lymphoid tissue (GALT) (Adapted from reference [25].

6. Body Defense Enhancement

The PCFs are associated with different antimicrobial activities. As it can be observed in **Figure 3**, the PG includes MPs, MDPs, and MTPs, which all confer passive defense on human gut against a wide range of pathogenic microbes. In addition, paraprobiotics contain other immunological components, such as growth factors, S-layer proteins, cytoplasmic extracts including LTAs, CpG-DNA motifs, WPS, EPS, and other molecules, which can modulate the immune system. Such anti-infective fragments can be presented as "novel nutraceutical ingredients" for functional foods because they boost specific and nonspecific body defenses.

6.1. Anti-Microbial and Anti-Viral Activities

The S-layer proteins of paraprobiotics have been found to inhibit the adhesion of enteropathogenic bacteria such as *Clostridium perfringens* [26], *Escherichia coli* O157:H7, *Salmonella* Typhimurium [27], and *Clostridium difficile*, *Shigella* sp., and *Salmonella* sp. [28] to mucus and/or intestinal epithelial cells in a competitive way. The literature reveals that if the S-layer proteins of lactobacilli are damaged or removed, the adhesion activity to the host epithelial cells is compromised [29]. They can also displace, inhibit the binding of enterobacterial toxins to their receptors, especially to mucous and Caco-2 cell surface, competitively [29]. While the CpG-DNA motifs are known to activate epithelial cells through interaction with TLRs (see **Figure 3**) for sinus, influenza, meningoencephalitis, and virus infections, unspecified DNA motifs have demonstrated a higher level of antagonistic activity against bacterial and viral infections [18]. PCFs contain a number of immunostimulatory DNA motifs.

Moreover, paraprobiotics have been found to contain about 2939 to 3381 different host defense peptides (HDPs) [30]. In general, the HDPs are known to influence the host response to infections in several ways, including direct antimicrobial killing, promoting wounds healing, production of cytokines, modulation of chemokines, enhance angiogenesis [31], limiting inflammation, anti-sepsis, enhancing vaccine responses, and regulating metabolism [32]. We also believe that HDPs are deeply involved in the activation of neutrophils. Activated neutrophils are reported to have a persistent presence with a continued recruitment and activation throughout the interrupted healing process in chronic wounds as well [33]. Equally important, the bacteriocins from various probiotics have attracted considerable attention in the area of food preservation [34] and may be characterized into "novel nutraceutical ingredients" too, because they can inhibit foodborne pathogenic and spoilage bacteria.

6.2. Anti-Allergic, Anti-Mutagenic, Anti-Inflammatory, and Anti-Infectious Activities

Immunostimulatory sequences of DNA (ISS-DNA) and their synthetic sequences of oligodeoxynucleotides (ODNs) are the 2 identified structures of immunostimulatory DNAs [35]. They are derived from immunobiotic LAB and deeply involved in host defenses against tumor, inflammations, and other infections. Elsewhere, the DNA motifs of CpG-ODN have been reported to suppress allergen-specific acidophilic respiratory tract inflammation in murine models of bronchial asthma and also improves airway hyperreactivity [36]. Generally, the DNA motifs are known to be involved in the immunostimulatory events and are still gaining an advanced understanding of their competence in regulating immune networks in the intestine through cytokines induction (see **Figure 3**). Someday, the production of PCFs as "novel nutraceutical ingredients" would be playing a significant role in the development of immunogenic foodstuffs, immunogenic feeds, and immunogenic drugs. These would have immediate effects to the prevention and protection of the host against all kinds of allergies, infectious-, autoimmune-, and inflammatory-diseases. Although it seems to be accepted that EPSs could not yield significant commercial products on their own [37], they are still being recommended for health-promoting effects, such as immune-modulating activities, anti-tumor, promising cholesterol-lowering activity, and prebiotic activity [38].

6.3. Immunomodulating Effects

Various PGs, HDPs or their peptides have been found to act as immunomodulating compounds. By employing the probiotic CFT^{TM}, MPs, MDPs, MTPs were found to enhance phagocytosis and modulate the proliferation and/or differentiation of human peripheral blood lymphocytes [18]. For example, pure MDPs of *L. rhamnosus* V have been shown to stimulate the immune system predominantly by phagocytosis. This instance demonstrates that each peptide may have various biological activities. The mechanisms by which the PCFs exert their immunomodulatory effects, including individual thousands of HDPs [30], are stipulated in **Figure 3**. It can be clearly seen that the immunomodulatory effects are all related to the NLRs and TLRs. Our experimental and clinical data also confirmed that PCFs are strongly involved in the regulation of Th1/Th2 paths of the immune response as per **Figure 4**. Efficiency of the PCFs is predetermined by their ability to have an impact on different links of innate and adaptive immunity, both specific and non-specific links, thus be able to control and coordinate the immune response by Th1 or Th2 pathways, depending upon the immune status of the body.

Most of research confirmed that stimulation with paraprobiotics as MAMPs, certainly activated the macrophages, DCs, epithelial cells, and trans-membrane receptors, thereby, induces an increase in the production level of a variety of cytokines, chemokines, and costimulatory molecules (**Figure 3**). They notify the host to breach in the mucosal barrier and turn the immune response at the site of infection [25]. Most of the cytokines (ILs, IFNs, TNFs are produced from either adherent T lymphocytes (macrophages and DCs) or non-adherent T lymphocytes. Depending on the exact combination and timing of clear signals that are coming from the epithelium and/or resident effector cells. For example, the DCs grow and respond differentially whereby their cytokines control the direction and types of the immune response to be initiated [25]. In our study, orally administered PCFs demonstrated the ability to induce the production of TNF and NK cells, as well as to regulate the synthesis of IL-4, IL-10, IL-12, and IFNs (like **Figure 3**, **Figure 4**, **Figure 6** & **Figure 7**). It should be known also that the *in vivo* results of cytokine production upon ingestion of probiotics has been found to vary greatly in both humans and

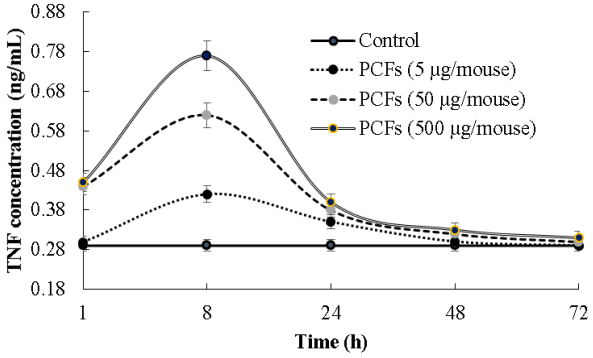

Figure 6. Murine serum TNF dynamics after oral administration of PCFs in mice.

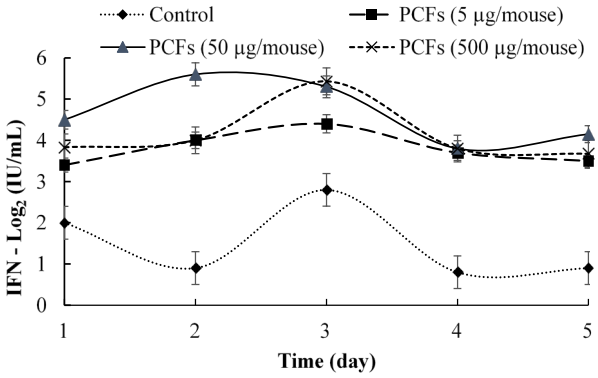

Figure 7. Interferon activity of PCFs in mice.

animals. They seemed to depend on the kinds of strain used and the experimental settings. TLR-9 is one of the PRRs which is highly involved in the recognition of un-methylated CpG DNAs, plentifully existent in bacterial genomes (see **Figure 3**).

Bacterial DNA motifs induces immunoglobulin secretion and triggers B-cell proliferation [39]. Compared to **Figure 3**, another research has also reported that the cytoplasmic fractions of paraprobiotics, which is mainly LTA, induce macrophages to release IL-6 and TNF-α, and increase the number of IgA (+) cells in the lamina propria of the small intestine [39]. In addition to DNA motifs, different peptides as part of PCFs have been found to inhibit the proliferation of *Staphylococcus aureus* and protected mice from infection of herpes and meningoencephalitis by stimulating T lymphocytes proliferation and phagocytosis cells, *in vivo* [18]. It is, therefore, thought that the continuous exposure of the PCFs to problematical antigens reduces the immune responses and normalizes the production of IgA and IgG antibodies by enhancing suppressor T cells. Consequently, these immunomodulating PCFs are qualified as health-corrective ingredients for functional foods, nutritional supplements, distinctive drugs, cosmetics, animal health products, and so forth.

7. Putative Protective Effects on Chronic Diseases and Infections

7.1. Anti-Carcinogenic Activities and Anti-Malaria Possibility

PCFs have demonstrated anti-proliferative activities with regards to the preclusion of cancer. Many experimental studies have suggested different mechanisms about the intake of most components found in the PCFs and their chemo-preventive role in various forms of cancer. For example, the anti-cancer effects of cellular components of lactobacilli have been attributed to: 1) binding of genotoxic carcinogens; 2) interaction with the immune system; 3) induction of apoptosis exerting anti-proliferative activity and differentiation of cancer cells; 4) butanol extract of *B. adolescentis* dose dependently precluded the development of colon cancer cells (Caco-2, HT-29, and SW480); 5) concentrated supernatants of *L. plantarum* precluded the rise of human promyelocytic cell line (HL-60 cells); 6) cell-bound EPS of *L. acidophilus* exerted anti-tumor effects on HT-29 cells; 7) probiotical cell walls and/or cytoplasm extracts indicated significant anti-proliferative activities against cancer [40]. However, it should be acknowledged here that research on the anti-carcinogenic effects using PCFs is challenging due to the diverse etiologies of cancers. Interestingly, PCFs of *L. delbrueckii* subsp. *bulgaricus* LB86 VCIM-B5788 have been approved in Ukraine, and the formulation is still being used there as a supportive medication for anti-cancer treatments [18].

The impression that paraprobiotic DNA motifs may exhibit strong anti-tumor effects against tumors of the same type was first made by reference [41] in 1984. Both the CpG-ODN and non-CpG-ODN DNA-motifs are each found to play a different role, depending on the paraprobiotic strains used. However, there is enough evidence that unmethylated CpG-DNA sequences and ODNs are all involved in the prevention and treatment of cancers, allergies, infectious diseases, and inflammatory diseases [42]. Immunosuppressive ODNs, on the other hand, have been shown to suppress the symptoms of multiple sclerosis, sepsis, endotoxic shock, and the formation and proliferation of tumor cells, because they suppress the activity of $CD8^+$ T cells and B cells [35]. Therefore, paraprobiotics with immunosuppressive oligodeoxynucleotide mutated in CpG sequence (ISM-ODN) are indebted for their identified cures. The literature revealed that the ISM-ODN binds strongly to high-mobility

group box (HMGB) and prevent activation of the immune system [35]. Thus far, several AT-ODNs that have been discovered made an immense influence in the medical field and others are still being considered in drug developments for malaria [35] [43], an infectious disease.

EPS has been found to protect mice from severe influenza viral infections [23]. In addition, EPSs have reliable immunostimulatory effects such as anti-tumorigenesis, activation of macrophages, induction of cytokines, and B-cell mitogenic activities [23]. Consequently, PCFs seem to be effective in braking the replication of the cancer cells; however, they (PCFs) do not bother the normal tissues. Equally important, the mitochondria has been found to play a vital protagonist in the thriving and dying of cancer cells [40] [44]. Ever since that discovery, the amount of mitochondrial membrane potential ($\Delta\Psi m$) loss has then started gaining popularity worldwide as this is known to be associated with the initiation of apoptotic mitochondrial pathway on cancer cells [40]. While the mechanisms of $\Delta\Psi m$ loss are still complicated, the exposure of PCFs to cancer cells is required to breakdown the $\Delta\Psi m$ through the modulation of mitochondrial signal pathway. Our research has shown that oral administration of PCFs in mice manifested a high level of the TNFs within 8 h of ingestion (**Figure 6**). In addition, the production level of critical IFNs was raised 2.5 - 3 times as compared to the control (**Figure 7**). Overall, the PCFs demonstrated the ability to induce the production of NK cells, TNFs, and to regulate the synthesis of IL-4, IL-10, IL-12, IFNs, and neutrophils [18]. Fascinatingly, higher dosages of PCFs did not always result in higher effectiveness.

7.2. Putative Anti-Parasitogenic Activity

The oral administration of paraprobiotic *L. casei* or its spent culture supernatant has been shown to reduce *Trichinella spiralis* infection in the animal intestine from 32 - 44% [20]. In the same study, a reduction of *T. spiralis* larvae in muscle tissues was also observed. The protective mechanisms of action appear to depend on the colonization of the intestine by cell fragments of lactobacilli, macrophage processing of the PCFs or probiotic cell lysates, and activation of T and B-cells through IL-2 production. The active components of PCFs are the prime objects in exerting the health benefits to the host, and their gut penetrability is straightforward as elucidated in **Figure 5**. The reality here is that the PCFs are being transported and absorbed through the intestine. With regards to anti-parasitogenic activity, the PCFs may act on the pathogenic parasites for humans by the production of IL-10 from CD4+ T-cells. However, further investigations by using PCFs as nutraceutical ingredients are still needed and be validated *in vivo*.

8. Use of PCFs as Heath Corrective Ingredients

The use of paraprobiotics in the prevention and treatment of a dramatic list of acute and chronic diseases, immune deficiencies, as well as infectious diseases such as viral (flu, hepatitis C), bacterial (bronchitis), asthma, fungal etiology, chronic fatigue, fibromyalgia, and colonic cancer etiology has been featuring lately in gastroenterology at an increasing rate, indisputably. To date, the cellular fragments that make paraprobiotics and indeed, the PCFs particularly suitable for diverse industrial applications have been recently evolving while others continue to be carefully investigated. These include their GRAS status, dairy fermenting properties as starter culture supplements and/or stabilizers, immunomodulating properties, ability to evoke mucosal and systemic immune responses against associated antigens, coaggregation with pathogens and competitive exclusion, decreasing the luminal pH, provision of specific compounds such as bacteriocins or HDPs, adhesion to mucus and/or intestinal epithelial cells in a competitive nature against enteropathogenic bacteria, and many more.

PCFs may be marketed in many different formats. In the future, it will be attractive to see paraprobiotics in the form of PCFs and packaged as: 1) Nutritional supplements, and can be included in liquid foods , powder foods, gels, creams, gummies, pills and sprays; 2) specific drugs that are intended to relieve or mitigate, cure, or prevent infections and diseases; 3) food additives intended for beverages, ready-to-eat foods, and functional foods; 4) direct-feed lysates meant to be fed to animals that produce the foods and/or those which requiring support for health issues (including pets); 5) starter-culture stabilizers for many nourishers; 6) cosmetics meant for retarding the significant effects of aging, and so on. It is, however, expected that small amounts of PCFs may be added to foods and labeled as such, because these natural nutraceutical ingredients have a track record for improving health.

The shelf stability of one unique product with PCFs known as “Del-Immune V®” has been measured and found to be as from 3-5 years [18]. In foods (for example, bread, ice-cream or cheese) or beverages, if the PCFs

are to be incorporated, they are expected to last for much longer time when compared to their counterpart viable probiotics which require refrigeration while at the same time to be consumed in an adequate amount to take advantage of the most testified health benefits. At this point, it is essential to stress that, the form in which the PCFs may be formulated and consumed is an economical advantage to the host. With the PCFs, product preparation, storage, transit time, and expiration date are secondary factors in how much live probiotic cells are missing during ingestion or application. For instance, improper handling and storage can kill viable bacteria in yogurts, and it is difficult to trace or know how the product was handled prior to purchase or use. With a PCFs-yogurt, these kinds of concerns are not valid to anybody to utilize such a product. The same principle applies to many other possible PCF formulations, depending on the selected strains and the intentions. High post-fermentation acidification is another controversial situation that affects the sensory quality of yogurt; that is characterized by a good flavor, smooth texture and suitable viscosity. With PCF-yogurt, post-fermentation acidification would not be a serious issue and the actual sensory characteristics be retained as time goes.

9. Concluding Remarks

It is encouraging to demonstrate the need of the PCFs as "novel nutraceutical ingredients" in the paraprobiotic prevention and treatment cases. The kinds of paraprobiotic treatments would depend on the selected bacterial strain, host response, and the intended purpose. For example, the preferred PCFs for patients with common allergic diseases (food allergy, bronchitis, hay fever, and asthma) are somehow different to that of rectal cancer, gastroenteritis or IBD patients. In allergic diseases, one of the highest target of PCFs (extracts of *L. rhamnosus* V) is DCs due to their ability to polarize T cell responses. Allergic diseases often result from exaggerated Th2 type immune responses. For the prevention of allergic diseases per se, the PCFs positively modulate T cell polarization into an increase in Th1 cell and CD4+CD25+ regulatory T cell responses, primarily by modulating DC functions. Other conclusions embrace that the PCFs accelerate normalization of humoral immunity, increase resistance to bacterial and viral infections, increase the activity of NK cells and phagocytosis, increase production of IL-1, IL-2, and TNFs, significantly increase in T-lymphocytes or T cells production, normalize the contents of IgA and IgGs, prevent side effects after antibiotics and cyclophosphamide usage, induce cytosuppression, and significantly reduce the toxic side effects. The combined effects of PCFs appear to be specific in their individual actions whereby making the completion of physicochemical- and/or radio-therapy possible without disruption. In addition, the PCFs offer many advantages for use as active health corrective ingredients in functional foods, beverages, starter cultures, nutritional supplements, specific drugs, animal feeds or health products, cosmetics, chronic wound treatments, and many more including prospects of formulating the anti-malaria drugs.

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References

[1] FAO/WHO (2002) Guidelines for the Evaluation of Probiotics in Food. http://www.who.int/entity/foodsafety/publications/fs_management/probiotics2/en

[2] Taverniti, V. and Guglielmetti, S. (2011) The Immunomodulatory Properties of Probiotic Microorganisms beyond their Viability (Ghost Probiotics: Proposal of Paraprobiotic Concept). *Genes and Nutrition*, **6**, 261-274. http://dx.doi.org/10.1007/s12263-011-0218-x

[3] Saito, T. and Kitazawa, H. (2005) Recent Tendency of Immunogenics Research on Lactic Acid Bacteria. *Bulletin of Japan Dairy Technical Association*, **55**, 34-44. (In Japanese)

[4] Kitazawa, H., Tohno, M. Shimosato, T. and Saito, T. (2008) Development of Molecular Immunoassay System for Probiotics via Toll-Like Receptors Based on Food Immunology. *Animal Science Journal*, **79**, 11-21. http://dx.doi.org/10.1111/j.1740-0929.2007.00491.x-i1

[5] Kitazawa, H., Villena, J. and Alvarez, S. (2013) Probiotics: Immunobiotics and Immunogenics. CRC Press, Boca Raton.

[6] Lebeer, S., Vanderleyden, J. and De Keersmaecker, C.J. (2010) Host Interactions of Probiotic Bacterial Surface Molecules: Comparison with Commensals and Pathogens. *Nature Reviews Microbiology*, **8**, 171-184. http://dx.doi.org/10.1038/nrmicro2297

[7] Kleerebezem, M., Hols, P., Bernard, E., Rolain, T., Zhou, M., Siezen, R.J. and Bron, P.A. (2010) The Extracellular Bi-

ology of the Lactobacilli. *FEMS Microbiology Reviews*, **34**, 199-230. http://dx.doi.org/10.1111/j.1574-6976.2009.00208.x

[8] Kaji, R., Kiyoshima-Shibata, J., Nagaoka, M., Nanno, M. and Shida, K. (2010) Bacterial Teichoic Acids Reverse Predominant IL-12 Production Induced by Certain Lactobacillus Strains into Predominant IL-10 Production via TLR2-Dependent ERK Activation in Macrophages. *The Journal of Immunology*, **184**, 3505-3513. http://dx.doi.org/10.4049/jimmunol.0901569

[9] Clancy, R. (2003) Immunobiotics and the Probiotic Evolution. *FEMS Immunology and Medical Microbiology*, **38**, 9-12. http://dx.doi.org/10.1016/S0928-8244(03)00147-0

[10] Underhill, D.M. and Ozinsky, A. (2002) Toll-Like Receptors: Key Mediators of Microbe Detection. *Current Opinion in Immunology*, **14**, 103-110. http://dx.doi.org/10.1016/S0952-7915(01)00304-1

[11] Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C. (2008) Genes and Molecules of Lactobacilli Supporting Probiotic Action. *Microbiology and Molecular Biology Reviews*, **72**, 728-764. http://dx.doi.org/10.1128/MMBR.00017-08

[12] Hornung, V., Ablasser, A., Charrel-Dennis, M., *et al*. (2009) AIM2 Recognizes Cytosolic dsDNA and Forms a Caspase-1-Activating Inflammasome with ASC. *Nature*, **458**, 514-518. http://dx.doi.org/10.1038/nature07725

[13] Ouwehand, A.C. (2007) Antiallergic Effects of Probiotics. *The Journal of Nutrition*, **137**, 794S-797S.

[14] Hemmi, H., Takeuchi, O., Kawai, T., *et al*. (2000) A Toll-Like Receptor Recognizes Bacterial DNA. *Nature*, **408**, 740-745. http://dx.doi.org/10.1038/35047123

[15] Kawai, T. and Akira, S. (2011) Toll-Like Receptors and their Crosstalk with other Innate Receptors in Infection and Immunity. *Immunity*, **34**, 637-650. http://dx.doi.org/10.1016/j.immuni.2011.05.006

[16] Beutler, B.A. (2009) TLRs and Innate Immunity. *Blood*, **113**, 1399-1407. http://dx.doi.org/10.1182/blood-2008-07-019307

[17] Rhee, S.H. (2011) Basic and Translational Understandings of Microbial Recognition by Toll-Like Receptors in the Intestine. *Journal of Neurogastroenterology and Motility*, **17**, 28-34. http://dx.doi.org/10.5056/jnm.2011.17.1.28

[18] Del-Immune, V. (2014) Del-Immune V®. http://www.delimmune.com/research

[19] Tohno, M. and Kitazawa, H. (2013) Molecular Immunoassay Systems for Probiotics via Pattern Recognition Receptors. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics*: *Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 54-88. http://dx.doi.org/10.1201/b15532-5

[20] Humen, M.A., Benyacoub, J., Minnaard, J., *et al*. (2013) Immunobiotics and Immunity against Parasites. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics: Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 194-214. http://dx.doi.org/10.1201/b15532-9

[21] Stadelmann, B., Merino, M.C., Persson, L. and Svärd, S.G. (2012) Arginine Consumption by the Intestinal Parasite Giardia Intestinalis Reduces Proliferation of Intestinal Epithelial Cells. *PloS One*, **7**, e45325. http://dx.doi.org/10.1371/journal.pone.0045325

[22] Cerutti, A. and Rescigno, M. (2008) The Biology of Intestinal Immunoglobulin A Responses. *Immunity*, **28**, 740-750. http://dx.doi.org/10.1016/j.immuni.2008.05.001

[23] Makino, S., Ikegami, S., Nagai, T. and Yamada, H. (2013) Immunogenics: Extracellular Polysaccharides Reduce the Risk of Infection. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics: Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 376-397. http://dx.doi.org/10.1201/b15532-16

[24] Yoda, K., Miyazawa, K., Harata, G. and He, F. (2013) Immunobiotics and Antiviral Immunity. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics*: *Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 169-193. http://dx.doi.org/10.1201/b15532-8

[25] Christensen, H.R. and Frøkiær, H. (2007) Immunomodulating Effects of Lactic Acid Bacteria. In: Shetty, K., Paliyath, G., Pometto, A.L. and Levin, R.E., Eds., *Functional Foods and Biotechnology*, CRC Press, New York, 435-471.

[26] Matsumoto, M., Tani, H., Ono, H., Ohishi, H. and Benno, Y. (2002) Adhesive Property of *Bifidobacterium Lactis* LKM512 and Predominant Bacteria of Intestinal Microflora to Human Intestinal Mucin. *Current Microbiology*, **44**, 212-215. http://dx.doi.org/10.1007/s00284-001-0087-4

[27] Chen, X.Y., Xu, J.J., Shuai, J.B., Chen, J.S., Zhang, Z.F. and Fang, W.H. (2007) The S-Layer Proteins of *Lactobacillus Crispatus* Strain ZJ001 Is Responsible for Competitive Exclusion against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. *International Journal of Food Microbiology*, **115**, 307-312. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.11.007

[28] Xue, C., Zhang, L., Li, H., *et al*. (2013) Functionality of the S-Layer Proteins from Lactobacillus in the Competitive against Enteropathogens Infection. *European Food Research and Technology*, **236**, 249-255. http://dx.doi.org/10.1007/s00217-012-1871-z

[29] Lee, Y.K., Puong, K.Y., Ouwehand, A.C. and Salminen, S. (2003) Displacement of Bacterial Pathogens from Mucus

and Caco-2 Cell Surface by Lactobacilli. *Journal of Medical Microbiology*, **52**, 925-930. http://dx.doi.org/10.1099/jmm.0.05009-0

[30] Klein, G., Schanstra, J.P., Hoffmann, J., Mischak, H., Siwy, J. and Zimmermann, K. (2013) Proteomics as a Quality Control Tool of Pharmaceutical Probiotic Bacterial Lysate Products. *PloS One*, **8**, e66682. http://dx.doi.org/10.1371/journal.pone.0066682

[31] Bowdish, D.M., Davidson, D.J., Scott, M.G. and Hancock, R.E. (2005) Immunomodulatory Activities of Small Host Defense Peptides. *Antimicrobial Agents and Chemotherapy*, **49**, 1727-1732. http://dx.doi.org/10.1128/AAC.49.5.1727-1732.2005

[32] Afacan, N.J., Janot, L.M. and Hancock, R.E.W. (2013) Host Defense Peptides: Immune Modulation and Antimicrobial Activity *in Vivo*. In: Hiemstra, P.S. and Zaat, S.A.J., Eds., *Antimicrobial Peptides and Innate Immunity*, Springer, Basel, 321-358.

[33] Valdez, J.C., Ramos, A.N., Fernández, D., *et al*. (2013) Probiotics and their Potential Use in Wound Treatment. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics: Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 298-335. http://dx.doi.org/10.1201/b15532-13

[34] Liu, W., Zhang, L., Yi, H., *et al*. (2014) Qualitative Detection of Class IIa Bacteriocinogenic Lactic Acid Bacteria from Traditional Chinese Fermented Food Using a YGNGV-Motif-Based Assay. *Journal of Microbiological Methods*, **100**, 121-127. http://dx.doi.org/10.1016/j.mimet.2014.03.006

[35] Shimosato, T. and Kitazawa, H. (2013) Immunogenics: Immunostimulatory Oligodeoxynucleotides from Probiotics. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics: Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 336-350. http://dx.doi.org/10.1201/b15532-14

[36] Ramaprakash, H., Shibata, T., Duffy, K.E., *et al*. (2011) Targeting ST2L Potentiates CpG-Mediated Therapeutic Effects in a Chronic Fungal Asthma Model. *The American Journal of Pathology*, **179**, 104-115. http://dx.doi.org/10.1016/j.ajpath.2011.03.032

[37] Jolly, L., Vincent, S.J.F., Duboc, P. and Neeser, J.R. (2002) Exploiting Exopolysaccharides from Lactic Acid Bacteria. *Antonie van Leeuwenhoek*, **82**, 367-374. http://dx.doi.org/10.1023/A:1020668523541

[38] Nakajima, H., Suzuki, Y. and Hirota, T. (1992) Cholesterol Lowering Activity of Ropy Fermented Milk. *Journal of Food Science*, **57**, 1327-1329. http://dx.doi.org/10.1111/j.1365-2621.1992.tb06848.x

[39] Galdeano, C.M., Dogi, A.C. and Perdigón, G. (2013) Difference in the Signals Induced by Commensal or Probiotic Bacteria to the Gut Epithelial and Immune Cells. In: Kitazawa, H., Villena, J. and Alvarez, S., Eds., *Probiotics*: *Immunobiotics and Immunogenics*, CRC Press, Boca Raton, 36-53. http://dx.doi.org/10.1201/b15532-4

[40] Wang, S., Zhang, L., Fan, R., *et al*. (2014) Induction of HT-29 Cells Apoptosis by Lactobacilli Isolated from Fermented Products. *Research in Microbiology*, **165**, 202-214. http://dx.doi.org/10.1016/j.resmic.2014.02.004

[41] Tokunaga, T., Yamamoto, H., Shimada, S., *et al*. (1984) Antitumor Activity of Deoxyribonucleic Acid Fraction from *Mycobacterium Bovis* BCG. I. Isolation, Physicochemical Characterization, and Antitumor Activity. *Journal of the National Cancer Institute*, **72**, 955-962.

[42] Klinman, D.M. (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nature Reviews Immunology*, **4**, 249-259. http://dx.doi.org/10.1038/nri1329

[43] Yanai, H., Chiba, S., Ban, T., *et al*. (2011) Suppression of Immune Responses by Nonimmunogenic Oligodeoxynucleotides with High Affinity for High-Mobility Group Box Proteins (HMGBs). *Proceedings of the National Academy of Sciences*, **108**, 11542-11547. http://dx.doi.org/10.1073/pnas.1108535108

[44] Nicholls, D.G. and Ward, M.W. (2000) Mitochondrial Membrane Potential and Neuronal Glutamate Excitotoxicity: Mortality and Millivolts. *Trends in Neurosciences*, **23**, 166-174. http://dx.doi.org/10.1016/S0166-2236(99)01534-9