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Potential Molecular and Cellular Mechanisms Underlying the Anti-Inflammatory and Anti-Tumor Properties of Probiotics: Our Experience

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Abstract

A probiotic is defined as a *"live microorganism which when administered in adequate amounts confers a health benefit on the host"*. There is a growing interest in probiotics within the scientific community, with consumers, and in the food industry. Thus, a large body of literature exploring the mechanisms of action of probiotic strains and their effects on human health is actually available.

In the last twenty years, our research group has been pledged to study the cellular and molecular mechanisms underlying the beneficial effects of selected strains of probiotics.

Here, we retrace the most significant stages of our research on probiotics, reporting, in particular, *in vitro* and *in vivo* findings regarding their anti-inflammation and anti-cancer properties.

Introduction

Many bacterial strains have been developed as natural "probiotics", most prominently those of the *Lactobacillus* and *Bifidobacterium* genera. These microbes have been used to treat *Clostridium* infection [1]; inflammatory diseases such as obesity, diabetes, and inflammatory bowel disease (IBD) [2]; and neurological conditions such as anxiety, depression, and autism spectrum disorder [3], among other pathologies. Probiotics are known to exert beneficial effects by directly signaling with the human host through chemical or physical means or by altering the composition and metabolism of the gut microbiota [4]. However, the genetic and molecular mechanisms by which natural probiotics evolved to act are poorly understood [5, 6]. Recent reviews have focused on engineering bacteria to target cancer [7, 8] or genetically modifying the endogenous gut microbiota *in situ* [9, 10, 11].

In the present article, we retrace the most significant stages of our research on probiotics, reporting, in particular, *in vitro* and *in vivo* findings regarding their anti-inflammation and anti-cancer properties.

Anti-inflammatory and anti-tumor properties of probiotics

In the '90s, we started to study and then we reported the efficacy of probiotic organisms in the treatment of pouchitis by evaluating their ability either to increase tissue levels of the anti-inflammatory cytokine IL-10, or to decrease, to levels present in control pouches, proinflammatory cytokines (i.e. TNF-α, IFN-γ, and IL-1α), inducible nitric oxide synthase, and matrix metalloproteinase activity, thus suggesting a mechanism of action to explain the efficacy of this therapeutic regime in pouchitis [12].

At the same time, we investigated the apoptotic effect *in vitro* of sonicated preparations of selected strains of lactic acid bacteria (LAB) on normal and tumor human lymphocytes [13]. Interestingly, incubation with bacterial samples led to a relevant time-dependent apoptotic cell death of human T-leukemia Jurkat cells but not normal human peripheral blood lymphocytes. *Lactobacillus brevis* and *Streptococcus thermophilus* samples were more efficient in inducing Jurkat apoptosis than other probiotics. In an attempt to characterize the mechanisms underlying these effects, we found that the apoptotic death-inducing ability of *S. thermophilus* preparations could be attributed to the ability of high levels of neutral sphingomyelinase activity to generate in Jurkat cells relevant amounts of ceramide, a known apoptotic death messenger. On the other hand, our results showed that apoptosis induced by *L. brevis* samples could also be associated with high levels of arginine deiminase activity, which in turn was able to downregulate polyamine synthesis in Jurkat cells. Arginine deiminase enzyme catalyzes the catabolism of arginine thus deprivating ornithine decarboxylase activity of the common substrate and, consequently, affecting the biosynthesis of polyamines known to be overexpressed in neoplastic cells. The obtained results led us to suggest that these two functional probiotics, *S. thermophilus* and *L. brevis*, possessed peculiar biochemical characteristics underlying the

anti-cancer effects observed *in vitro* and mainly related to the activity of neutral sphingomyelinase and arginine deiminase, respectively.

In this context, considering that functional probiotics may prevent *Helicobacter pylori* infection, our group assessed also whether oral administration of *L. brevis* was able to affect *H. pylori* survival in the human gastric mucosa [14]. The effects of *L. brevis* administration on polyamine biosynthesis in gastric biopsies from *H. pylori*-positive patients was also evaluated. *L. brevis* treatment led to a reduction in the urea breath test delta values, suggesting a decrease in intragastric bacterial load. Of note, *L. brevis* induced a decrease in gastric ornithine decarboxylase activity and polyamine levels. Our data supported the hypothesis either that *L. brevis* treatment decreased *H. pylori* colonization, thus reducing polyamine biosynthesis, or, alternatively, the arginine deiminase activity following *L. brevis* administration caused arginine deficiency, preventing polyamine generation from gastric cells.

Several years ago, when intracellular pathways leading from membrane receptor engagement to apoptotic cell death were still poorly characterized, we investigated the intracellular signalling generated after cross-linking of CD95 (Fas/Apo-1 antigen), a broadly expressed cell surface receptor whose engagement results in triggering of cellular apoptotic programs [15]. We reported evidence that crosslinking of CD95 with DX2, a functional anti-CD95 monoclonal antibody, resulted in the activation of a sphingomyelinase in promyelocytic U937 cells, as well as in other human tumor cell lines and in CD95-transfected murine cells, as demonstrated by induction of *in vivo* sphingomyelin hydrolysis and generation of ceramide. Our data, showing that CD95 cross-linking induced sphingomyelin breakdown and ceramide production through an acidic sphingomyelinase, provided the first information regarding early signal generation from CD95, and was considered relevant in defining the biochemical nature of intracellular messengers leading to apoptotic cell death. In 1995, we also showed evidence about the role of multiple phospholipid hydrolysis in Fas/APO-1 signalling [16]. Our efforts to identify among different effectors which one belongs to the apoptotic pathway, pointed toward the sequential PC-PLC/acidic sphingomyelinase activation as a key step for the propagation of the death signal. Then, we also investigated the expression and function of Fas (CD95/APO-1) on human T lymphocytes resident within the intestinal lamina propria, a major site of antigen challenge and persistent lymphocyte activation [17]. Taken together, our results provided the first evidence of a role for ceramide-mediated pathways in normal immunoregulation.

Considering that a markedly reduced mucosal alkaline sphingomyelinase activity has been found in premalignant and malignant intestinal epithelia and in ulcerative colitis tissue, our group reported evidence regarding the alkaline sphingomyelinase activity in feces from healthy subjects and colorectal adenocarcinoma patients to correlate it with the enzyme activity in intestinal tissues [18]. The findings, with potential implications for cancer biology and perhaps also for the design of clinical test, suggested that the fecal sphingomyelinase activity could really reflect the human intestinal mucosa enzyme level and could represent a new marker for human colorectal adenocarcinoma, mainly taking into account its early appearance in intestinal neoplasms.

We also showed evidence that probiotic-derived neutral sphingomyelinase mediates the beneficial effect of probiotics in inflammatory bowel disease. The results suggested that induction of immune cell apoptosis could be a mechanism of action of some probiotics, and that neutral sphingomyelinase-mediated ceramide generation contributed to the therapeutic effects of probiotics [19].

The involvement of bacterial SMase was also studied in skin aging and inflammation [20, 21]. Health benefits of probiotics have been established by several studies in animals and humans and the scientific literature shows that the clinical uses of probiotics are broad and are open to continuing evaluation. The most common microorganisms used as probiotics are strains of LAB, which are gram-positive, nonsporing, catalase-negative organisms that are devoid of cytochromes and of nonaerobic habit, but are aerotolerant, acid-tolerant, and strictly fermentative; lactic acid is the major end product of sugar fermentation. Particular attention is paid to specific species of LAB, including Lactobacilli and Bifidobacteria, that are part of the intestinal microbiota. Most probiotics are included in foods or dietary supplements and are aimed at functioning in the intestine. However, even if gastrointestinal tract has been the primary target, it is becoming evident that other conditions not initially associated with the gut microbiota might also be affected by probiotics. We reported a significant increase in skin ceramide levels in healthy subjects after treatment *in vivo* with a cream containing a preparation of *S. thermophilus*. The presence of high levels of neutral sphingomyelinase activity in this organism was shown to be responsible for the observed increase of stratum corneum ceramide levels, thus leading to an improvement in barrier function and maintenance of stratum corneum flexibility [22].

Considering that a reduced amount of total ceramides could be responsible for functional abnormalities of the skin of atopic dermatitis (AD) patients, we also investigated the effects of the topical administration of a *S. thermophilus*-containing cream on ceramide levels of stratum corneum from AD patients [23]. A 2-week application of the cream, containing a sonicated preparation of the lactic acid bacterium *S. thermophilus*, in the forearm skin of 11 patients led to a significant and relevant increase of skin ceramide amounts, which could have resulted from the sphingomyelin hydrolysis through the bacterial sphingomyelinase. Moreover, in all patients the topical application of our experimental cream also resulted in the improvement of the signs and symptoms characteristic of AD skin (i.e. erythema, scaling, pruritus).

We also investigated the effects of the topical treatment of a *S. thermophilus*-containing cream on ceramide levels of stratum corneum of healthy elderly women [24]. The ceramide levels, transepidermal water loss and capacitance were evaluated on stratum corneum sheets from the forearms of healthy female subjects treated with a base cream or the same cream containing a sonicated preparation of the lactic acid bacterium *S. thermophilus*. The probiotic treatment led to significant and relevant increase of stratum corneum ceramide levels. Moreover, the hydration values of the treated forearm of each subject were significantly higher than control sites. Altogether, these results suggested that the experimental cream was able to improve the lipid barrier and to increase a resistance against ageing-associated xerosis.

A great attention was focused by our group on the interaction between host and probiotics which may have anti-inflammatory properties and immunomodulatory activities. In this context, in the past we investigated the effect of a *Bifidobacterium infantis* extract on the abnormal apoptosis of HaCaT cells induced by soluble factors (IFN-γ and CD95 ligand) released by human T-lymphocytes *in vitro* activated with anti-CD3/CD28 mAbs or the mitogen PHA (phytohemoagglutinin) [25]. Of note, the bacterial extract treatment was able to totally prevent T lymphocyte-induced HaCaT cell apoptosis *in vitro*. The mechanism underlying this inhibitory effect was suggested to depend on the ability of the *B. infantis* extract to significantly reduce anti-CD3/CD28 mAbs and mitogen-induced T-cell proliferation, IFN-γ generation and CD95 ligand release. Our results represented an experimental basis for a potential therapeutic approach mainly targeting the skin disorders-associated immune abnormalities.

Anti-inflammatory effects of *L. brevis* extracts were also analysed by our group on periodontitis patients [26]. The involved mechanisms *in vitro* on activated macrophages were also investigated. Eight healthy subjects and 21 patients with chronic periodontitis were enrolled to analyze the effect of *L. brevis*-containing lozenges on periodontitis-associated symptoms and signs. Before and after the treatment, the patients received a complete periodontal examination. Saliva samples, collected before and after treatment, were analyzed for metalloproteinase and NOS activity, IgA, PGE_2 and IFN-γ levels. The treatment led to the total disappearance or amelioration of all analyzed clinical parameters in all patients. This was paralleled to a significant decrease of nitrite/nitrate, PGE_2, matrix metalloproteinase, and IFN-γ levels in saliva samples. Overall, our results suggested that the effects of *L. brevis* could be attributed to the high level of arginine deiminase activity which prevented nitric oxide generation by competing with NOS for the common substrate arginine. Our findings give further insights into the knowledge of the molecular basis of periodontitis and have a potential clinical significance, giving the experimental ground for a new innovative, simple and efficacious therapeutic approach of periodontal disease.

In a recent article [27], we report our experience on fractional CO_2 laser resurfacing providing the results of a new post-operative topical treatment with an experimental cream containing probiotic-derived active principles potentially able to modulate the inflammatory reaction associated to laser-treatment. The experimental cream containing *S. thermophilus* was administered post-operatively for 2 weeks to 42 consecutive patients who were treated with fractional CO_2 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.

Manufacturing may affect the efficacy and safety of probiotics

Variability in probiotic manufacturing may affect their properties, with potential implications for their 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 [28, 29].

Recently, we have shown that the VSL#3 formulations manufactured in the USA and Italy have a different effect on tumor cell lines and wound healing [30, 31]. The same discrepancies between VSL#3 formulations manufactured at different sites have been reported either by Biagioli et al. in animal models of IBDs [32] or by Trinchieri et al. in HIV-infected subjects [33]. Zacarias et al. have recently reported how technological processing of the probiotic strain *B. lactis* INL1 affected its functionality in inducing inhibitory activity against pathogens [34].

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.

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 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.

Competing interests

The authors declare that they have no competing interests.

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