

## **Probiotic Features of Lactic Acid Bacteria, Isolated from Artisanal Italian Dairy Products: A Positive Role in Wellbeing and Health and a Promising Experimental Design**

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**Received:** November 30, 2017; **Published:** January 03, 2018

### **Background and Significance**

The increased consumers' interest on the positive role of food in wellbeing and health underscores the need to determine new probiotic microorganisms [1].

Traditional dairy products manufactured with natural starter cultures are generally accepted by customers for their elevated nutritional and sensory quality. However, little is known on the role of these products in supplying microorganisms able to stably colonize the human gut and exert probiotic effects. Probiotics are defined by the Food and Agricultural Organization and the World Health Organization as "live microorganisms which when administered in adequate amounts, confer a beneficial health effect on the host" [2]. The evidence for the beneficial role of the gut microbiota in health and disease is mounting. In this respect, it is recognized that perturbations of the intestinal micro flora may, in turn, lead to a range of diseases, including inflammatory immune diseases such as Crohn's disease, obesity, cancer and infectious diseases [3-6]. Moreover, recent investigations have suggested a significant contribution of the gut microbiota in the regulation and impairment of energy homeostasis, thereby causing metabolic disorders, such as metabolic endotoxemia, insulin resistance and type 2 diabetes [7]. Thus, gut microbiota seems to exert a great variety of functional properties affecting human physiology and pathology [8].

In adults, the human microbiota contain  $\sim 10^{14}$  bacteria comprising between 1000 and 1150 different species [9] that exist in a mutualistic relationship with the host and aid in digestion, modulation of the immune system and manufacture of essential nutrients. Additionally restoring the current balance is very important because the commensal bacteria are significant source of vitamins, amino acids and lipid homeostasis and alternation in the levels of these metabolites might have an influence on the immune system [10]. Therefore, therapeutic manipulations of intestinal bacteria by selectively altering the beneficial versus detrimental species by probiotics and or prebiotics administration could reverse the inflammatory responses and restore mucosal homeostasis [11].

Given that, it is unlikely that a single probiotic strain may be sufficient to redress the imbalance of gut micro flora, and a more effective approach may be to use a cocktail of strains from a number of genera. This will pose a challenge in terms of identification of the most suitable mixes and the technological hurdles that will have to be overcome to produce stable probiotic mixes containing anaerobic organisms [12]. It is increasingly clear that the efficacy of a given probiotic strain is greatly dependent on the background microbiota of each individual, as well as the target human populations and specific strain properties.

### **Objectives**

1. As a first step, in this study we will evaluate the role of a fresh Pasta Filata cheese of the Alto Molise area called "Stracciata", made from milk of cows fed only by grazing and with local dry forages, as a source of potentially probiotic microorganisms.
2. Analyses of physiological and biochemical traits and molecular characterization by sequencing of the 16S rRNA gene will be carried out on all bacteria isolated from fresh Pasta Filata cheese.
3. Given that in the duodenal loop, probiotics bacteria are challenged by toxic detergent-like compounds such as bile salts and the bile tolerance is an important criterion in the selection of probiotic strains to be used as dietary supplements, transmembrane electrical potential ( $\Delta\Psi$ ) assays will be monitored in different stress conditions.
4. Antioxidant system activity and Glutathione disulphide (GSSG)/reduced Glutathione (GSH) ratio will be measured in each bacterial strain.

5. In addition, to identify anti-inflammatory probiotics, the ability of bacterial isolates to stimulate IL-10 and TNF- $\alpha$  production by human peripheral blood mononuclear cells (PBMC) will be determined *in vitro*.
6. Finally, identifying strains and performing microbial collection of probiotic strains will offer an interesting possibility for the future development of highly functional probiotic foods with health-promoting properties.

## Research Design and Methods

### Isolation of potentially probiotic bacteria

The dominant strains belonging to bacterial groups known to comprise probiotic strains, i.e. lactic acid bacteria (LAB) and propionic acid bacteria (PAB) present in the cheese from a single producer will be isolated by using MRS and SL, respectively. Plates will be incubated at 37°C for both bacterial groups. To prevent the growth of strictly anaerobic microorganisms, lactic acid bacteria will be allowed to grow in aerobiosis. Colonies selected on the basis of morphology will be purified on the same culture media and characterized at the genotype level by Rep-PCR with the GTG5 primer [13].

The bacteria isolated from cheese that exhibited identical profiles will be analysed by Multilocus Sequence Typing (MLST). Oligonucleotides will be supplied by Diatech (Italy). Sequencing will be carried out at Eurofins Genomics (Germany). The identification of the amplification products will be based on BLAST analysis at NCBI and sequences were compared by using MEGA 6.06 software.

### Transmembrane electrical potential ( $\Delta\Psi$ ) assays

In this study, the cytoplasmic membrane depolarization effect of bile acids on different prebiotic strains will be determined using the membrane potential-sensitive dye DiSC<sub>3</sub> probe [14].

### Antioxidant enzyme activities

The activities of SOD and catalase will be determined with bacterial cell homogenate (about 0.1 mg cell protein). SOD (E.C.1.15.1.1) activity will be measured by the inhibition of xanthine oxidase/cyt c system reaction as described by Kirby and Fridovich [15]. In this assay, xanthine oxidase, acting on xanthine in the presence of oxygen, generates superoxide anion, O<sub>2</sub><sup>-</sup> which reduces cyt c, and this reduction is inhibited by SOD. One enzymatic unit of SOD is the amount of enzyme required to inhibit the rate of reduction of cyt c by 50%; then the activity of SOD will be expressed as the percentage inhibition of the control reaction. Cell homogenate will be suspended at 25°C in 1.5 mL PBS buffer in the presence of Fe<sup>3+</sup>-cyt c (10  $\mu$ M) plus xanthine (50  $\mu$ M). The reaction will start with xanthine oxidase addition and the absorbance increase at 418 nm will be monitored.

Catalase (E.C.1.11.1.6) determination will be performed by a spectrophotometric assay based on the catalysed decomposition of H<sub>2</sub>O<sub>2</sub>, essentially according to Aebi [16]. The peroxide decomposition rate is directly proportional to the enzyme activity. To determine the catalase activity, cell homogenate will be suspended at 25°C in 1.5 mL PBS buffer in a quartz cuvette. After reading the initial absorbance at 240 nm, 100  $\mu$ L H<sub>2</sub>O<sub>2</sub> (final concentration 10 mM) will be added and the decrease in absorbance monitored. The slope of the absorbance versus time plot will be directly proportional to the activity of the sample and will be expressed as  $\Delta A_{240}/\text{min} \times 10^6$  cells.

### Glutathione disulphide (GSSG)/reduced glutathione (GSH) ratio measurement

GSH or GSSG will be assayed in the cell homogenate, according to Akerboom and Sies [17]. Briefly, GSH in the presence of methylglyoxal (2 mM) and glyoxalase I (6 e.u.) will be specifically converted into S-lactoyl-GSH which could be monitored directly at 240 nm; GSSG amount will be assayed in the same cuvette by measuring the stoichiometric conversion of NADPH (10  $\mu$ M) spectrophotometrically at 340 nm in the presence of glutathione reductase (1 e.u.).

### Bacterial co-culture and stimulation of PBMC

Overnight bacterial culture, grown under limited aeration at 37°C in MRS media, will be centrifuged at 9000g for 10 minutes, and the cell pellets will be suspended to approximately 10<sup>7</sup> CFU/mL in PBS (pH 7.00). Volumes of 20  $\mu$ L of the thawed bacterial suspensions will add to the PBMC resulting in a bacteria-to-cell ratio of approximately 10:1. PBS containing 20% glycerol will be used as a negative (non-stimulated control). After 24h of co-incubation at 37°C in air with 5% CO<sub>2</sub> the culture supernatants will be collected, clarified by centrifugation and stored -20°C until subsequent cytokine analysis.

### Cytokines quantifications by ELISA

The ability of bacterial isolates to stimulate IL-10 and TNF- $\alpha$  production by peripheral blood mononuclear cells (PBMC) will be determined by commercial kits.

### Statistical analysis

All the data presented will be given as means  $\pm$  SE. The significance of the results compared to the control will be tested using Student's t-test; \*significant differences ( $\alpha = 0.01$ ).

### Conclusions

Probiotics have been proposed as preventive and therapeutic measures in order to restore the healthy microbiota composition and function of the GI tract.

Studies on the natural occurrence of probiotic bacteria specifically adapted to traditional cheeses are opportune with the ultimate aim to optimize their use for conferring health-promoting properties to these products.

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Volume 14 Issue 2 February 2018

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