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Traditional dairy products can supply beneficial microorganisms able to survive in the gastrointestinal tract

Carmela Amadoro[a], Franca Rossi[b,*], Maria Luigia Pallotta[a], Maurizio Gasperi[a], Giampaolo Colavita[a,b]

[a] Department of Medicine and Health Science "V. Tiberio", University of Molise, Campobasso, Italy
[b] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Diagnostic Laboratory, Isernia, Italy

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ABSTRACT

Little is known about the role of traditional dairy products in naturally supplying beneficial microorganisms able to survive in the human gastrointestinal tract (GIT). To investigate this aspect, a fresh artisanal Pasta Filata cheese was administered daily to 18 healthy children, 3–6 years of age, for seven days. Counts and type of lactic acid bacteria (LAB) and propionic acid bacteria (PAB) were carried out on the cheese and children's faeces before and after cheese consumption.

In most cases, statistically significant increases of presumptive LAB were observed after seven days from suspension compared to values before and at the end of consumption.

Based on repetitive element palindromic PCR (rep-PCR) genotyping, six cheese isolates were identical to faecal isolates. Identity was confirmed by sequencing regions of *clp*P and *rpo*D genes for LAB and *pep*N and *pro*W genes for PAB.

Among those cheese isolates *P. freudenreichii* S-1-P, *L. plantarum* S-2-2 and *L. helveticus* S-2-6 stimulated the production of high interleukin 10 (IL-10) and low tumor necrosis factor alpha (TNF-α) levels by peripheral blood mononuclear cells (PBMC). Therefore they could exert anti-inflammatory effects *in vivo*.

Results suggested that traditional dairy products should be more efficiently exploited as a natural source of health-promoting microorganisms.

1. Introduction

According to the expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP), scientific evidence supports the beneficial effects on health of fermented dairy products containing live microbes. These beneficial effects consist in a reduced risk of the following: type 2 diabetes, insulin resistance, weight gain over time, mortality, high levels of blood triglycerides and cholesterol and high systolic blood pressure (Hill et al., 2014; Zheng et al., 2015).

However, more investigations are needed to distinguish the contribution to these health-promoting effects of the living microorganisms from that of the food matrix. For this reason, at the moment such foods can be defined as "containing live and active cultures" or "containing probiotics", if they supply microorganisms proven to be effective in human trials (Hill et al., 2014).

For both food categories the recommendation of an adequate amount of microorganisms, i.e. at least 1×10^9 CFU per serving, is affirmed, in accordance with the recommended intake for probiotics of the Food and Agricultural Organization of the United Nations and World Health Organization (FAO/WHO) (Hill et al., 2014; FAO/WHO, 2002).

Traditional dairy products supply a highly diverse microbiota comprising a multiplicity of species of lactic acid bacteria (LAB) and dairy propionibacteria (PAB) with well recognized probiotic functions (Bertazzoni Minelli et al., 2004; Foligné et al., 2010). The demonstration that these bacterial groups, when supplied with these products, are able to survive in GIT, could lead to the recognition of the probiotic nature of this food category.

However, still little is known on the ability of traditional fermented dairy products (part of the diet for many communities in the world) to supply beneficial bacteria able to survive transit in the gastrointestinal tract (GIT) when ingested with the product.

Until now the health promoting effects exerted by the bacteria from traditional dairy products were investigated only indirectly, since bacteria were first isolated and then characterized *in vitro* and *in vivo* (Mahasneh & Abbas, 2010). Only one study, in which bacteria were

* Corresponding author. Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Diagnostic Laboratory, C.da Breccelle, 86170, Isernia, Italy.
E-mail address: f.rossi@izs.it (F. Rossi).

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discriminated to the strain level by Randomly Amplified Polymorphic DNA PCR (RAPD-PCR) and identified by 16S rRNA gene sequencing, that is very little discriminant at the intra-species level, investigated the influence of traditional food on the instauration of particular microbial components in GIT (Albesharat, Ehrmann, Korakli, Yazaji, & Vogel, 2011).

Therefore, the present study was conceived to investigate whether traditional dairy products can have a functional role by naturally supplying beneficial bacteria able to survive in GIT and exert health promoting effects. The demonstration that this category of products, characterized by a high microbial biodiversity, promotes consumer's health thanks to the activity of their natural microbiota would constitute an incentive to their safeguard and inclusion in everyday diet.

As a test product a fresh Pasta Filata cheese called “Stracciata”, typical of the Molise region in Central Italy, was chosen. The cheese has been administered to children, 3–6 years of age, and LAB and PAB were isolated from both cheese and faeces. Molecular techniques adequate to discriminate bacteria to the strain level were developed and allowed the identification of bacterial strains from Stracciata cheese that survived in GIT. Their ability to exert beneficial effects was investigated by analyzing their immunostimulation and immunomodulation capacity with respect to production stimulation of anti-inflammatory and pro-inflammatory cytokines interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) by peripheral blood mononuclear cells (PBMC).

2. Materials and methods

2.1. Study design and sample collection

The Stracciata cheese used in this study was provided by a dairy manufacturer which adheres to a local dairy consortium (I Formaggi del Tratturo, Agnone, Italy) committed to transforming only milk produced in the Alto Molise district from cows fed with local forages with natural whey cultures prepared from the previous manufacturing process.

Eighteen children aged 3 to 6, all living in the small town of 1380 inhabitants where the dairy products are manufactured, were enrolled in the study. They were selected after interviewing the parents of 46 children of the same age living in the town. A total of twenty-nine children were interested in taking part to the study but only 18 of them were able to confirm that the children had not been consuming traditional dairy products for the whole month prior to the study and that they appreciated the Stracciata cheese, so that they could eat the required amount without difficulty.

All participants followed a Mediterranean dietary regime comprising heat treated milk, wheat bread and sweet bakery products, pasta with tomato sauce and olive oil, cooked fresh meat, legumes, fruit and various vegetables.

Mothers were asked to let their children eat Stracciata cheese portions of about 50 g every day for seven days and not to administer probiotics or fermented food during the study. The cheese used came from two production batches manufactured on day 0 and day 4 (batches 1 and 2, respectively) from the beginning of the study. Sterile plastic containers were given to the mothers for the collection of the children's faeces at home before cheese consumption (control), at the end of the seven days of consumption (day 7), and after seven (day 15) and fifteen (day 21) days from suspension. They were also asked to ensure that the children did not eat cheese from the same dairy from day 8 until day 21 and to keep faecal samples refrigerated until being collected for microbiological analyses to be carried out on the same day. Faecal samples were labelled by mothers and brought to a single collection point. Stracciata cheese samples from the same manufacturing batches of those administered to children were transported in refrigerated conditions and analysed on the day of collection and a second time after 3 days of refrigerated storage for counts of presumptive LAB and PAB.

2.2. LAB and PAB isolation

Ten grams of the cheese were homogenized in 90 mL of sterile peptone solution (9 g L^{-1} NaCl, 1 g L^{-1} casein peptone from Biolife Italiana, Milan, Italy). Serial dilutions were plated in duplicate on MRS agar and sodium lactate agar (SLA) (Rossi, Capilongo, & Torriani, 1996), media and incubated anaerobically in jars containing Anaerocult (Merk Millipore, Vimodrone, Italy), at 37 °C and 30 °C, respectively.

Bacteria isolation from faeces was done by weighing 1 g of sample and adding 1 mL of sterile peptone water. This suspension was serially diluted and plated in duplicate on the same media reported above.

All colonies of different appearance were isolated from each cheese or faecal sample by two subsequent streaks on the same medium.

2.3. DNA extraction

Genomic DNA was obtained from the bacterial isolates by alkaline extraction as follows: the cell pellet obtained by centrifugation from 1 mL of fresh culture was re-suspended in 200 μL of 1 g L^{-1} NaOH and 100 mL L^{-1} Triton-X-100 solution. After 1 h incubation at room temperature the cell suspension was centrifuged at 8000 rpm for 5 min, the supernatant was removed and the pellet was re-suspended in 200 μL of 10 mmol L^{-1} Tris/HCl, pH 8.0. The supernatant obtained from this suspension was used for rep-PCR and for gene targeted PCR.

2.4. PCR protocols

Repetitive element palindromic PCR (rep-PCR) was carried out with the GTG_5 primer as described by Versalovic, Schneider, de Bruijn, and Lupski (1994). PCR tests targeted on specific genes were carried out with the primer pairs reported in Table 1. The PCR reactions contained 0.5 μmol L^{-1} primer in 1 × EmeraldAmp GT PCR Master Mix (DiaTech, Milan, Italy). For dairy propionibacteria sterile dimethylsulfoxide (DMSO) was added to the PCR reactions at 100 mL L^{-1} final concentration. Primer pairs were designed to work at an annealing temperature of 50 °C. The PCR program included initial denaturation at

Table 1
Primer pairs used in this study, respective annealing sites and size of the amplicon.

Labels	Sequence 5'→3'*	Positions	Target
cpu	GGYGAACGBGCYTAYGA	721315–721298[a]	A 356 bp region of the *clp*P gene of *Lactobacillus* spp.
cpd	TGDCCTTGNGCWCCACC	720959–720975	
rpdu	AABACYTTDCCNACYTCTTC	1774300–1774315[a]	A 721 bp region of the *rpo*D gene of *Lactobacillus* spp.
rpdd	AAYGAYCCHGTNCGDATGTA	1775021–1775002	
NU1	GCTGTGCCGCTA	477–488[b]	A 877 bp region of the *pep*N gene of dairy propionibacteria
D1	AVGTGATSCCGTCGAAGYT	1354–1335	
725′	TGGGCCGGGTCGGT	1–14[c]	A 915 bp region of the *pro*W gene of *Propionibacterium freudenreichii* subsp. *freudenreichii*
723′	GCCGGCCCGCCGA	915–903	

*According to the IUPAC code, the ambiguous primer positions have the following meaning: Y (C, T), V (A, C, G), D (A, G, T), W (A, T), H (A, C, T), S (C, G).

[a] Nucleotide positions referred to the *L. plantarum* WCFS1 genome, Acc. n. NC_004567, locus tags lp_0786 and lp_1962.

[b] Nucleotide positions referred to the *pep*N sequence of *P. jensenii* R3, Acc. n. AM184104.1.

[c] Nucleotide positions referred to the *pro*W gene of *P. freudenreichii* subsp. *freudenreichii* LMG 16415, Acc. n. AM110698.1.