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

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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

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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.

94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and elongation at 72 °C for 1 min, except for the cpu/cpd primer pair, for which the elongation time was of 30 s. Final extension was done at 72 °C for 5 min. The amplification products were separated on a 15 g L^{-1} agarose gel stained with 1 mL L^{-1} GelRed (Biotium, DiaTech) in 1× TAE buffer (80 mmol L^{-1} Tris/acetate, 2 mmol L^{-1} EDTA, pH 8.0) at 120 V.

2.5. Genetic profile comparison

The genetic profiles obtained by rep-PCR were compared by the PyElph 1.4 software for gel image analysis and definition of the phylogenetic relationships on the basis of a distance matrix (Pavel & Vasile, 2012). This program transforms the genetic profiles in series of 1 and 0 scores according to the presence or absence of bands in all the observed band positions. Distance values are obtained by subtracting the similarity percentage matrix, calculated by the Dice coefficient, to 100 times the matrix of ones. A dendrogram was generated by UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

2.6. Selection of molecular markers for isolate comparison

The molecular markers suitable to differentiate *Lactobacillus* strains at the intra-species level were chosen among 13 conserved genes, namely *clp*P, *com*X, *dna*A, *dna*J, *dna*K, *gro*EL, *hrc*A, *rpo*A, *rpo*D, *rpo*E, *ssb* and *tuf*, after conducting the following analysis for each candidate gene: *i* orthologs of each gene were searched in the genome of the species considered as representative of main sub-groups of the *Lactobacillus* genus (Rossi, Zotta, Iacumin, & Reale, 2016); *ii* their nucleotide sequences were aligned by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) in order to evaluate the possibility of designing genus-specific primers and, *iii* if good primers could be designed, the region comprised between the primer annealing sites was used in BlastN analyses (https://blast.ncbi.nlm.nih.gov/), run for each species considered, in order to evaluate the intra-species discriminating power of the molecular marker examined. Among genes that permitted the design of *Lactobacillus*-specific primers, *clp*P and *rpo*D exhibited the highest intra-species sequence diversity. Therefore, these genetic markers were chosen for the comparison of isolates with highly similar rep-PCR profiles from cheese and from faeces. For PAB primers amplifying a region of the aminopeptidase N (*pep*N) gene and the *pro*W gene (Rossi, Busetto, & Torriani, 2007; Rossi, Gatto, Marzotto, & Torriani, 2006) were used to compare strains. Sequences, positions and length of the amplification products for the primer pairs used in this study are reported in Table 1.

2.7. Sequence accession numbers

Sequences obtained in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers KY471272 (*Enterococcus faecalis* S-2-1, *rpo*D), KY471276 (*E. faecalis* S-2-1, *clp*P), KY471270 (*Lactobacillus plantarum* S-2-2, *rpo*D), KY471275 (*L. plantarum* S-2-2, *clp*P), KY471273 (*L. casei* S-2-3, *rpo*D), KY471277 (*L. casei* S-2-3, *clp*P), KY471271 (*Pediococcus pentosaceus* S-2-4, *rpo*D), KY471274 (*P. pentosaceus* S-2-4, *clp*P), KY856949 (*L. helveticus* S-2-6, *rpo*D).

2.8. Cytokine production assay

Cytokine production stimulation was analysed by using PBMCs (HuPBMNC, Stemcell Technologies, Voden Medical Instruments Spa., Peschiera Borromeo, Italy) which were adjusted to the concentration of 10^6 cells mL^{-1} in RPMI-1640 medium (Sigma Aldrich) supplemented with 0.75 glutamine mmoles L^{-1} and 100 mL L^{-1} fetal bovine serum in 6-well culture plates. Bacterial strains were added to PBMCs in numbers of 7 log CFU mL^{-1}. Bacterial cells were harvested from 1 mL of fresh culture by centrifugation at 8000 rpm for 10 min, washed once with sterile Dulbecco's phosphate buffered saline (DPBS) (8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 1.15 g L^{-1} disodium hydrogen phosphate and 0.2 g L^{-1} potassium hydrogen phosphate, pH 7.2) and re-suspended in 1 mL of DPBS. The number of bacterial cells was adjusted by measuring the OD 600 nm of fresh cultures and referring to a calibration curve constructed for each strain. Incubation of PBMCs in the presence of bacteria was carried out for 24 h in air with 5% CO_2 at 37 °C.

DPBS containing 200 g L^{-1} glycerol was used as a negative (non-stimulated) control. After incubation cell cultures were collected and centrifuged to obtain clear supernatants that were stored at −20 °C until cytokine analysis. Negative controls were represented by non-stimulated cell-cultures. The presence of cytokines in the cell culture supernatant was assayed in triplicate by Enzyme-linked Immunosorbent Assay (ELISA) with the Human IL-10 and TNF-α ELISA MAX Standard (BioLegend, Campoverde, Milan, Italy) according to the instructions. The number of cytokines was determined by OD measurement in a Victor3 1420 Multilabel Counter (Perkin Elmer Italia, Monza, Italy) microplate reader.

2.9. Statistical analysis of data

The significance of differences among faecal counts of presumptive LAB at different time intervals for each participant, as well as among induced cytokine levels, was analysed by the Student *t*-test.

3. Results and discussion

3.1. LAB and PAB presence in cheese and faeces

The Stracciata cheese was found to contain in average 7.25 ± 0.3 log CFU g^{-1} of presumptive LAB and 3.11 ± 0.5 log CFU g^{-1} of presumptive PAB on the day of production. The rather low initial number of bacteria in this Pasta Filata cheese was probably a consequence of the stretching phase of manufacture, which is carried out at temperatures of about 85 °C for some minutes. At day 3 the average numbers of presumptive LAB and PAB increased up to 8.69 ± 0.3 and 3.95 ± 0.1 log CFU g^{-1}, respectively. Therefore, the number of bacteria ingested daily ranged between 8.74 and 10.39 log CFU for presumptive LAB and between 4.95 and 5.65 log CFU for presumptive PAB. It can be noted that, for presumptive LAB, even the lower initial LAB numbers ingested were close to the requirement of 9 log CFU g^{-1} minimum dose recommended for probiotic dietary supply (Hill et al., 2014), while for presumptive PAB the supplied number was too low.

Analyses of faecal presumptive LAB and PAB could be carried out for all 18 participants at time 0 (before cheese consumption) and at the end of the Stracciata cheese consumption period (day 7), while six of the participants did not provide samples anymore at day 15, and only three participants, AL, AN and B, still provided faecal samples at day 21. This happened because only some participants could strictly follow the provided guidelines.

Counts of presumptive LAB in the faeces of the participants are reported in Table 2. Differences in faecal presumptive LAB counts between day 7 and day 0 were not statistically significant for any of the participants, except for participant PA ($P < 0.01$) who presented slightly lower values at day 7. At day 15 statistically significant increases of presumptive LAB counts compared to values before consumption were obtained for participants C ($P < 0.05$), AB, Z, 6 ($P < 0.01$), B and BR ($P < 0.001$). Increases in presumptive LAB counts at day 15 compared to day 7 were statistically significant for participants AL, B, C, G ($P < 0.05$), Z, 6 ($P < 0.01$), AB and BR ($P < 0.001$). For the participants AL, AN and B, who continued the study until day 21, the final numbers of presumptive LAB were 7.66 ± 0.03, 9.14 ± 0.03 and 9.55 ± 0.01 log CFU g^{-1}, respectively. The increases at day 21 for participant AL were statistically

Table 2
Numbers of presumptive LAB in faeces of the participants at day 0 (before cheese consumption); day 7 (after 7 days of consumption) and day 15 (after 7 days from suspension). Values are averages of two replicates with the respective standard deviations.

Day	Participant																	
	AL	AB	AN	B	BR	C	D	EM	F	G	J	K	N	P	PA	SA	Z	6
	log CFU g^{-1}																	
0	7.22 ± 0.01	6.34 ± 0.01	9.13 ± 0.04	9.24 ± 0.01	6.78 ± 0.03	7.86 ± 0.04	6.45 ± 0.03	6.11 ± 0.02	6.05 ± 0.03	7.51 ± 0.03	6.14 ± 0.02	9.28 ± 0.02	5.89 ± 0.04	7.45 ± 0.01	6.20 ± 0.01	7.57 ± 0.01	7.91 ± 0.03	7.41 ± 0.02
7	7.14 ± 0.02	6.47 ± 0.03	9.08 ± 0.05	9.30 ± 0.03	6.84 ± 0.02	7.91 ± 0.05	6.47 ± 0.04	6.00 ± 0.03	6.00 ± 0.01	7.46 ± 0.04	6.00 ± 0.04	9.25 ± 0.01	6.00 ± 0.03	7.50 ± 0.03	6.00 ± 0.01	7.69 ± 0.02	7.86 ± 0.04	7.32 ± 0.03
15	7.32 ± 0.02	7.84 ± 0.03	9.14 ± 0.01	9.64 ± 0.01	8.82 ± 0.03	8.57 ± 0.02	na	na	6.20 ± 0.06	7.78 ± 0.02	6.23 ± 0.04	na	na	na	na	7.79 ± 0.04	6.77 ± 0.05	8.80 ± 0.01

na; not available.

significant compared to values at day 0 ($P < 0.05$), 7 ($P < 0.01$) and 15 ($P < 0.05$). For participant B the increases at day 21 were statistically significant compared to those at day 0 ($P < 0.01$), 7 ($P < 0.05$) and 15 ($P < 0.01$).

Though even small increases in average counts of presumptive LAB at day 15 compared to day 7 resulted in statistical significance, a noticeable increase of more than 1 log CFU g^{-1} was observed only for participants AB, BR and 6 at day 15. Since for the same participants at day 7 the LAB counts were comparable to those before cheese consumption, Stracciata cheese might have introduced bacteria able to colonize GIT and increase in number. Some of the factors that might have influenced the capability of cheese bacteria to multiply in GIT are the competitiveness of the resident microbiota and diet variability, which might create an environment favourable or non-favourable to their survival. The observations suggest it should be determined whether the desirable effect of LAB increase could be extended to more subjects by supplying cheese with a higher number of autochthonous bacteria.

Presumptive PAB could be isolated only for participant C at day 15 and their number was 5.3 ± 0.01 log CFU g^{-1}, with a single colony type. The difficulty of isolating PAB from faeces was probably determined by the low specificity of the SLA growth medium. On the other hand, this was chosen because of its higher recovery of PAB compared to more selective formulations (Rossi et al., 1996). Based on such evidence, the necessity to improve PAB-specific media formulation to improve the isolation of these bacteria from faeces emerged. When supplied in numbers as low as those found in faeces in this study, PAB do not purportedly exert probiotic effects. Nevertheless, the results indicated their ability to survive in GIT when supplied with the cheese and their potential to exert beneficial activities if administered in higher amounts.

3.2. Rep-PCR results

Bacterial strains were isolated from cheese and from faeces. Cheese isolates were labelled with "S", that stands for "Stracciata", followed by the manufacturing batch of origin, 1 or 2, collected at day 0 and 4, respectively, from the beginning of the study. Faeces isolates were labelled with the participant tag, assigned by mothers in order to evaluate anonymously the results for their child at the end of the study, followed by the day of isolation, 0, 7, 15 or 21 since the beginning of the study and, separated by another dash, the isolate number. Only one presumptive PAB isolate was recovered from each source, S-1-P and C-15-6P, respectively.

Rep-PCR with the GTG_5 primer (Versalovic et al., 1994; Zunabovic, Domig, Pichler, & Kneifel, 2012), was adopted as genotyping method to eventually identify bacterial isolates from cheese with highly similar genotypes with bacteria from faeces. This method was used in a preliminary genotyping phase, aimed at isolate de-replication, from which 16 bacteria with unique genotypic profiles, among cheese isolates, and 93 bacteria with unique genotypic profiles, among faeces isolates, were obtained.

Results of genotypic profile comparison of the selected bacteria are shown in Fig. 1, where isolates from cheese are reported in red characters and isolates from faeces are reported in black characters. Duplicate profiles from isolates PA-7-4 and N-7-3 were included in the analysis in order to define a distance value at which to consider the isolates to be possibly identical.

It can be observed that the bacteria isolated from faeces before cheese consumption (day 0) were all distantly related from cheese isolates, while faecal isolates closely related to cheese isolates were obtained only after cheese consumption (days 7, 15 and 21), thus indicating that the latter could have been supplied by the Stracciata cheese.

A noticeable diversity in the intestinal presumptive LAB composition among the participants was observed, with bacteria from different

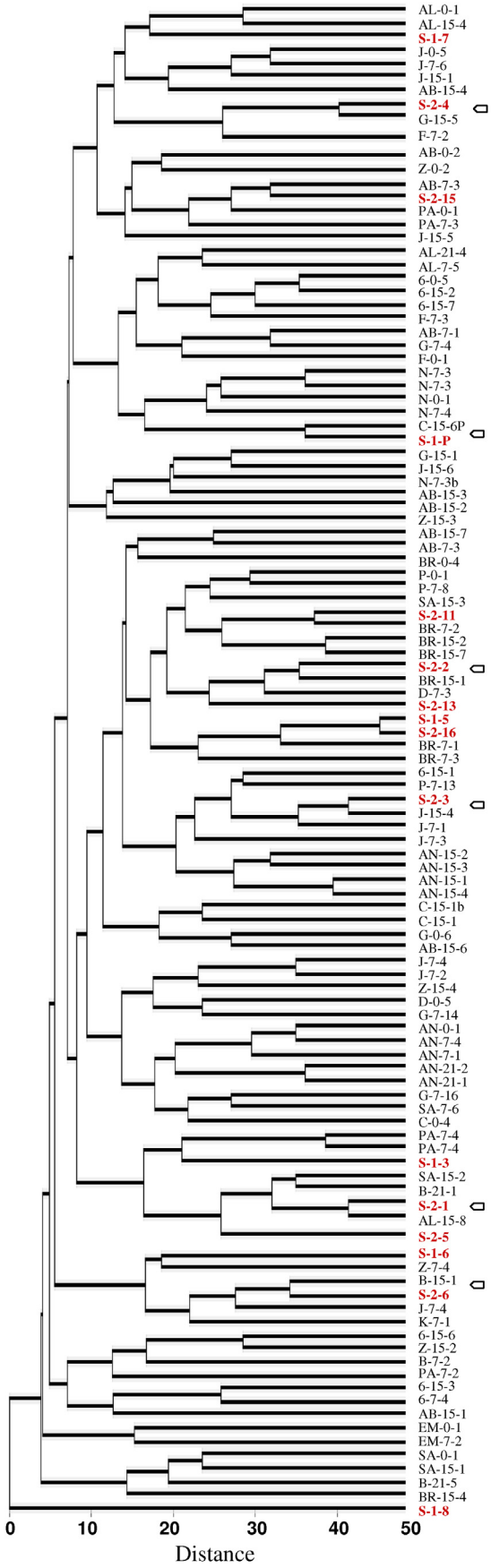

Fig. 1. Dendrogram constructed on the basis of rep-PCR profiles of presumptive LAB and PAB bacterial isolates from Stracciata cheese and faeces from children who consumed the cheese. Isolates from cheese were labelled with "S" followed by the cheese batch number, 1 or 2, collected at day 0 and 4, respectively, from the beginning of the study, and the isolate number. In the labels of faeces isolates the first letters represent the participant tag and are separated by a dash from the numbers 0, 7, 15 or 21, that indicate the day of sampling since the beginning of the study. The latter are, in turn, separated by a dash from the last number that indicates the individual isolate. Arrows indicate the isolate pairs that were used in *rpo*D and *clp*P sequence comparison.

individuals falling in distantly related clusters. This difference in species and strains that composed the resident gut microbiota can be one of the factors that could influence the colonization capacity of the bacteria supplied by cheese.

A diversification has been highlighted also within isolates obtained from the same individual and bacteria assigned to distantly related clusters have been obtained from each participant.

The isolates from Stracciata cheese and from faeces joined by distance values close to those of the duplicate isolates PA-7-4 and N-7-3 were considered to be possibly identical. Faecal isolates closely related to cheese isolates were chosen for molecular marker sequencing, to investigate if they could be considered the same bacterial strain. In Fig. 1 the isolate pairs composed by one isolate from cheese and one from faeces highly similar to each other, that were selected for this analysis, are indicated by arrows. Isolates B-21-1 and SA-15-2, closely related to isolates S-2-1 and AL-15-8, were also included. Only faeces isolates obtained at days 15 and 21 were considered for this purpose since they appeared to persist longer in GIT, at least in the short term. These could be isolated from seven participants, AL, B, BR, G, C, J and SA.

3.3. Molecular marker sequence comparisons

Sequencing of conserved genes *clp*P and *rpo*D, *pep*N and *pro*W genes for LAB and PAB, respectively, was carried out to compare isolates with highly similar genotypes obtained from cheese and from faeces.

The sequenced regions of the molecular markers *clp*P and *rpo*D were with no exception identical for the bacteria from cheese and from faeces with the most similar rep-PCR profiles. At the same time, the sequences obtained allowed the identification of isolates to the species level, as reported in Table 3.

The primer pairs designed for lactobacilli allowed to obtain amplification products also from *Enterococcus* spp. and *Pediococcus* spp., thus experimentally demonstrating that these primers can be used for identification and intra-species discrimination also of the latter bacterial genera. However, the primers targeted on *clp*P did not originate an amplification product from *L. helveticus* S-2-6 and from the most similar faecal isolate *L. helveticus* B-15-1, possibly due to strain-specific sequence divergence in the primer annealing site.

Among the cheese isolates found to be able to survive in GIT, *L. plantarum* BR-15-1 and *L. helveticus* B-15-1 were present at numbers approaching those recommended to exert probiotic effects, since they were isolated from faecal samples with a number of presumptive LAB close to 9 log CFU g^{-1}.

Notably, isolates with *rpo*D and *clp*P gene sequences identical to the cheese isolate *E. faecalis* S-2-1 were obtained from three participants, thus indicating a better colonization capacity of this isolate than the other bacteria that were found to survive in GIT.

Notably, all the bacterial biotypes that survived in GIT were mostly related to strains with demonstrated probiotic properties. In particular, the enterococcal isolates shared the highest alignment scores with the probiotic strain *E. faecalis* Symbioflor 1, included in a commercial probiotic product and used for more than 50 years without any report of infection (Fritzenwanker et al., 2013). However, the absence of virulence and other hazardous traits, as well as transmissible antibiotic

Table 3
Isolates from Stracciata cheese and children faeces with identical sequences of the molecular markers *rpo*D and *clp*P and microorganisms sharing highest alignment scores and sequence identity.

Cheese isolates	Faeces isolates	Database sequences with highest alignment scores and identity (%)	
		*rpo*D	*clp*P
S-2-1	AL-15-8, B-21-1, SA-15-2	*E. faecalis* str. Symbioflor 1 Acc. n. HF558530.1, 100%	*E. faecalis* str. Symbioflor 1 Acc. n. HF558530.1, 99%
S-2-4	G-15-5	*P. pentosaceus* wikim20 Acc.n. CP015918.1, 99%	*P. pentosaceus* ATCC 25745 Acc.n. CP000422.1, 99%
S-2-2	BR-15-1	*L. plantarum* MF1298 Acc. n. CP013149.1, 100%	*L. plantarum* MF1298 Acc. n. CP013149.1, 99%
S-2-3	J-15-4	*L. paracasei* CAUH35, Acc. n. CP012187.1, 99%	*L. paracasei* CAUH35, Acc. n. CP012187.1, 98%
S-2-6	B-15-1	*L. helveticus* CAUH18, Acc. n. CP012381.1, 99%	nd

nd; not determined.

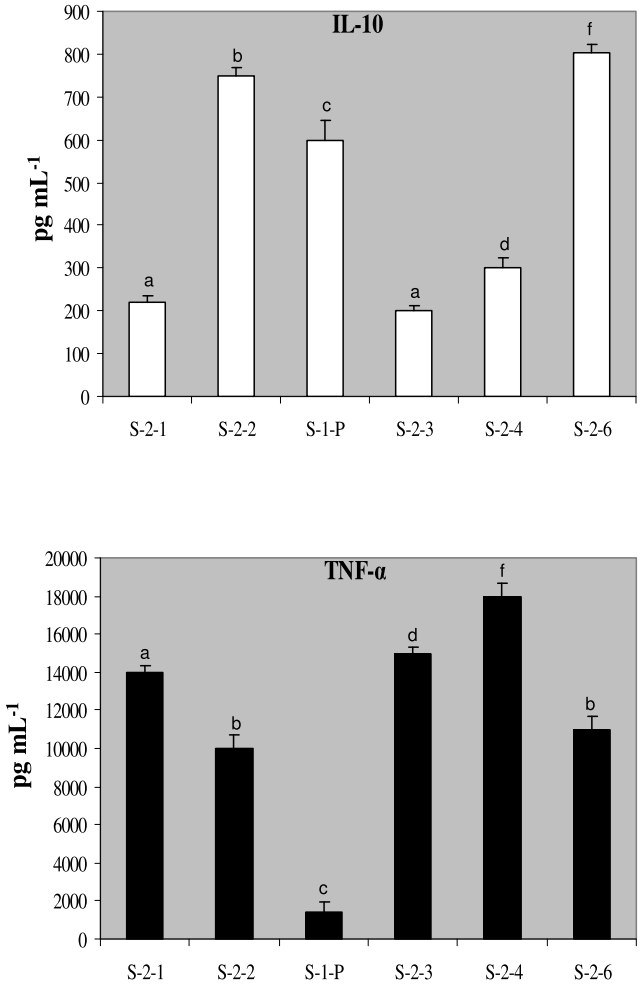

Fig. 2. IL-10 and TNF-α production by PBMC after stimulation with Stracciata cheese isolates *E. faecalis* S-2-1, *L. plantarum* S-2-2, *P. freudenreichii* S-1-P, *L. paracasei* S-2-3, *P. pentosaceus* S-2-4 and *L. helveticus* S-2-6. To the reported values those of the control were subtracted.

resistance encoding elements, must still be excluded experimentally for the Stracciata cheese isolates according to recently suggested procedures (Aspri, Bozoudi, Tsaltas, & Papademas, 2017).

Isolates S-2-2 and BR-15-1 showed highest alignment score and 100% identity of the *rpo*D sequence with five *L. plantarum* strains, comprising the probiotics *L. plantarum* MF1298 (Jensen, Grimmer, Naterstad, & Axelsson, 2012) and *L. plantarum* ZS 2058, a strain with high conjugated linoleic acid production ability (Chen, Xu, Ye, Chen, & Zhang, 2009). The *clp*P sequence was 99% identical to 26 database entries, among which the *clp*P ortholog of the above probiotic strains.

Isolates S-2-4 and G-15-5 were mostly similar to *P. pentosaceus* strain wikim20 from Korean kimchi (Lee et al., 2016), a food rich in living microorganisms and with numerous health promoting effects (Park, Jeong, Lee, & Daily, 2014).

Isolates S-2-3 and J-15-4 showed highest similarity of the *clp*P and *rpo*D genes only with *L. casei* CAUH35, a probiotic strain isolated from koumiss.

For the two dairy PAB isolates primers Nu1/D1 (Rossi et al., 2007) were used to amplify a *pep*N region found by BlastN analysis to contain variable nucleotide positions. Primer D1 was modified in order to better match the *pep*N sequences of *P. freudenreichii* (e.g. NC_014215.1, locus tag PFREUD_RS03975; NZ_CP010341, locus tag RM25_RS03795) that were published after the design of that consensus primer. The *pep*N region sequenced for isolates S-1-P and C-15-6P were 100% identical to each other and to those of the strains *P. freudenreichii* subsp. *shermanii* NCFB 853 (Acc. n. AM184106.1), *P. freudenreichii* subsp. *shermanii* PFREUDJS1 (Acc. n. LN997841.1) and CIRM-BIA1 (Acc. n. FN806773.1). The primer pair 725′-723' (Rossi et al., 2006) was used to amplify and sequence a region of the *pro*W gene (Acc. n. AM110698.1), that has a high intra-species variability based on BlastN analysis. The *pro*W sequences obtained for isolates S-1-P and C-15-6P were 100% identical to each other and to those of *P. freudenreichii* 16415 and *P. freudenreichii* subsp. *shermanii* CIRM-BIA1.

3.4. Cytokine production assays

Immunostimulation and immunomodulation by induction of cytokine production represent main mechanisms by which probiotics exert their beneficial functions (Dong, Rowland, & Yaqoob, 2012). In particular, IL-10 and TNF-α production stimulation by PBMCs was analysed in this study since these mediators play opposite roles in inflammatory responses and their levels and ratio are indexes of immunostimulation and immunomodulation, respectively, with corresponding *in vivo* effects (Dong et al., 2012). Indeed, IL-10 down-regulates the inflammatory response and induces an antibody-mediated immune response (Kekkonen et al., 2008), while TNF-α is a potent inducer of inflammatory molecules (Hehlgans & Pfeffer, 2005), so that their balance is relevant for the control of immune deviation (Salminen, Collado, Isolauri, & Gueimonde, 2009). Moreover, activation of IL-10 production by human PBMCs by bacterial strains can predict their potential anti-inflammatory effect *in vivo* (Foligné et al., 2007).

The cheese isolates *E. faecalis* S-2-1, *L. plantarum* S-2-2, *L. paracasei* S-2-3, *P. pentosaceus* S-2-4, *L. helveticus* S-2-6 and *P. freudenreichii* S-1-P, sharing identical molecular marker sequences with isolates from faeces, were tested for immunostimulation *in vitro*.

These isolates exhibited statistically different levels of induced production of IL-10 and TNF-α except for *E. faecalis* S-2-1 and *L. paracasei* S-2-3, in induced production of IL-10, and for *L. plantarum* S-2-2 and *L. helveticus* S-2-6 in induced production of TNF-α (Fig. 2).

IL-10 levels different for $P < 0.05$ were induced by *E. faecalis* S-2-1 compared to *P. pentosaceus* S-2-4, *E. faecalis* S-2-1 and *L. helveticus* S-2-6, and by *L. plantarum* S-2-2 compared to *P. freudenreichii* S-1-P and *L.*

helveticus S-2-6. Differences in IL-10 induced production significant for $P < 0.01$ were observed between *E. faecalis* S-2-1 and *P. freudenreichii* S-1-P, between *P. freudenreichii* S-1-P and *L. casei* S-2-3, *P. pentosaceus* S-2-4 and *L. helveticus* S-2-6, respectively, and between *P. pentosaceus* S-2-4 and *L. casei* S-2-3. All the other strain pairs differed for $P < 0.001$ in induced IL-10 levels.

Induced TNF-α levels differed for $P < 0.05$ between *E. faecalis* S-2-1 and *L. casei* S-2-3, for $P < 0.01$ between *E. faecalis* S-2-1 and *L. plantarum* S-2-2, *P. pentosaceus* S-2-4 and *L. helveticus* S-2-6, respectively, between *L. plantarum* S-2-2 and *P. freudenreichii* S-1-P, *L. casei* S-2-3 and *P. pentosaceus* S-2-4, respectively, and between *L. casei* S-2-3 and *P. pentosaceus* S-2-4 and *L. helveticus* S-2-6, respectively. All the other strain pairs differed for $P < 0.001$ in TNF-α induced levels.

L. plantarum S-2-2, *L. helveticus* S-2-6 and *P. freudenreichii* S-1-P showed a more intense stimulation of the anti-inflammatory cytokine IL-10 production and a lower TNF-α production stimulation compared to the other strains, thus showing that some of the bacteria supplied with dairy products might exert anti-inflammatory effects. In agreement with a study carried out on dairy PAB (Foligné et al., 2010), the *Propionibacterium freudenreichii* strain also induced relatively high IL-10 amounts and low TNF-α amounts. While the ability to stimulate IL-10 production was found to be variable for the strains of *P. freudenreichii* tested by Foligné et al. (2010), and was high for some, all strains induced a low level of TNF-α production.

Variation in the cytokine response induced by different bacterial strains was already reported and it was hypothesized that the ability to treat inflammatory bowel disease and prevent pouchitis by a mixture of eight probiotic strains was due to the upregulated production of IL-10. The main effect on IL-10 induction on dendritic cells was exerted by bifidobacteria (Gionchetti et al., 2003; Hart et al., 2004). The ability to induce high levels of IL-10 can be considered desirable also based on evidence that probiotic strains with this characteristic offered the best protection from acute 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis *in vivo* in mice and could be useful in the treatment of inflammatory diseases (Foligné et al., 2007; Kekkonen et al., 2008). Moreover, induction of IL-10 by probiotics was considered to be possibly responsible for the amelioration of symptoms of rheumatoid arthritis (Hatakka et al., 2003).

The relatively low induced levels of IL-10, coupled with higher induced levels of TNF-α, by *L. casei* S-2-3 was in agreement with the findings of Dong et al. (2012) on *L. casei* Shirota, while for the strain *L. plantarum* S-2-2 higher IL-10 and lower TNF-α production stimulation were observed in this study compared to *L. plantarum* NCIMB 8826. Results obtained for *E. faecalis* S-2-1 were discordant from those previously reported by Sparo, Delpech, Battistelli, and Basualdo (2014), who found higher levels of IL-10 and lower levels of TNF-α to be induced in PBMCs by a heat killed *E. faecalis* strains, though exposure to heat might have modified the immunogenic properties of those strains. Therefore, based on results from this study, variability in cytokine stimulation capacity within the specie *L. plantarum* and *E. faecalis* was highlighted. No data are available for comparison for *L. helveticus* S-2-6 and *P. pentosaceus* S-2-4.

4. Conclusions

This study indicated that traditional dairy products can directly contribute potential probiotics to the consumer. However, the ability of LAB and PAB of dairy origin to survive and to persist in GIT in the short term was observed in a minority of participants, so that further investigations are required to define an optimal dietary intake of the autochthonous bacteria supplied with the product and ensure more uniform outcomes. Consequently, an intervention on the manufacturing process is deemed necessary to introduce use of the most promising probiotic candidates isolated from the product as culture adjuncts.

The ability of the dairy bacteria to survive in GIT for periods longer than those tested in this study should be investigated in order to evaluate the long-term effects of the uptake of cheese natural microbial components.

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References

Albesharat, R., Ehrmann, M. A., Korakli, M., Yazaji, S., & Vogel, R. F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Systematic & Applied Microbiology, 34*, 148–155.

Aspri, M., Bozoudi, D., Tsaltas, D., & Papademas, P. (2017). Raw donkey milk as a source of *Enterococcus* diversity: Assessment of their technological properties and safety characteristics. *Food Control, 73*, 81–90.

Bertazzoni Minelli, E., Benini, A., Marzotto, M., Sbarbati, A., Ruzzenente, O., Ferrario, R., et al. (2004). Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. *International Dairy Journal, 14*, 723–736.

Chen, H., Xu, Q., Ye, Q., Chen, W., & Zhang, H. (2009). Bioconversion kinetics of conjugated linoleic acid by *Lactobacillus plantarum* ZS2058. *Weishengwu Xuebao, 49*, 174–179.

Dong, H., Rowland, I., & Yaqoob, P. (2012). Comparative effects of six probiotic strains on immune function *in vitro*. *British Journal of Nutrition, 108*, 459–470.

Foligné, B., Deutsch, S. M., Breton, J., Cousin, F. J., Dewulf, J., Samson, M., et al. (2010). Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced *in vitro* and *in vivo*. *Applied and Environmental Microbiology, 76*, 8259–8264.

Foligné, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., et al. (2007). Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. *World Journal of Gastroenterology, 13*, 236–243.

Food and Agricultural Organization of the United Nations and World Health Organization (2002). Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Fritzenwanker, M., Kuenne, C., Billion, A., Hain, T., Zimmermann, K., Goesmann, A., et al. (2013). Complete genome sequence of the probiotic *Enterococcus faecalis* Symbioflor 1 clone DSM 16431. *Genome Announcements, 1* e00165–12.

Gionchetti, P., Rizzello, F., Helwig, U., Venturi, A., Lammers, K. M., Brigidi, P., et al. (2003). Prophylaxis of pouchitis onset with probiotic therapy: A double-blind, placebo-controlled trial. *Gastroenterology, 124*, 1202–1209.

Hart, A. L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., et al. (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut, 53*, 1602–1609.

Hatakka, K., Martio, J., Korpela, M., Herranen, M., Poussa, T., Laasanen, T., et al. (2003). Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis – a pilot study. *Scandinavian Journal of Rheumatology, 32*, 211–215.

Hehlgans, T., & Pfeffer, K. (2005). The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: Players, rules and the games. *Immunology, 115*, 1–20.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology, 11*, 506–514.

Jensen, H., Grimmer, S., Naterstad, K., & Axelsson, L. (2012). *In vitro* testing of commercial and potential probiotic lactic acid bacteria. *International Journal of Food Microbiology, 153*, 216–222.

Kekkonen, R. A., Kajasto, E., Miettinen, M., Veckman, V., Korpela, R., & Julkunen, I. (2008). Probiotic *Leuconostoc mesenteroides* ssp. *cremoris* and *Streptococcus thermophilus* induce IL-12 and IFN-gamma production. *World Journal of Gastroenterology, 14*, 1192–1203.

Lee, S. H., Jung, M. Y., Park, B., Sung-Oh, S., Park, H. W., Choi, H. J., et al. (2016). Complete genome sequence of *Pediococcus pentosaceus* strain wikim 20, isolated from Korean kimchi. *Genome Announcements, 4*, 6.

Mahasneh, A. M., & Abbas, M. M. (2010). Probiotics and traditional fermented foods: The eternal connection (Mini-Review). *Jordan Journal of Biological Sciences, 3*, 133–140.

Park, K. Y., Jeong, J. K., Lee, Y. E., & Daily, J. W., 3rd (2014). Health benefits of kimchi (Korean fermented vegetables) as a probiotic food. *Journal of Medicinal Food, 17*, 6–20.

Pavel, A. B., & Vasile, C. I. (2012). PyElph - a software tool for gel images analysis and phylogenetics. *BMC Bioinformatics, 13*, 9.

Rossi, F., Busetto, M., & Torriani, S. (2007). Isolation of aminopeptidase N genes of food associated propionibacteria and observation of their transcription in skim milk and acid whey. *Antonie van Leeuwenhoek, 91*, 87–96.

Rossi, F., Capilongo, V., & Torriani, S. (1996). Confronto tra diversi terreni selettivi per la conta e l'isolamento dei batteri propionici in latte ovino. *Industria del Latte, 32*, 33–43.

Rossi, F., Gatto, V., Marzotto, M., & Torriani, S. (2006). A genetic insight into peptide and amino-acid utilization by *Propionibacterium freudenreichii* LMG 16415. *Current Microbiology, 52*, 464–468.

Rossi, F., Zotta, T., Iacumin, L., & Reale, A. (2016). Theoretical insight into the heat shock response (HSR) regulation in *Lactobacillus casei* and *L. rhamnosus*. *Journal of*

Theoretical Biology, 402, 21–37.
Salminen, S., Collado, M. C., Isolauri, E., & Gueimonde, M. (2009). Microbial-host interactions: Selecting the right probiotics and prebiotics for infants. *Nestlé Nutrition Institute Workshop, Pediatric Program, 64*, 201–213 discussion 213-7, 251-7.
Sparo, M., Delpech, G., Battistelli, S., & Basualdo, J.Á. (2014). Immunomodulatory properties of cell wall extract from *Enterococcus faecalis* CECT7121. *Brazilian Journal of Infectious Diseases, 18*, 551–555.
Versalovic, J., Schneider, M., de Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based Polymerase Chain Reaction. *Methods in Molecular and Cellular Biology, 5*, 25–40.
Zheng, H., Yde, C. C., Clausen, M. R., Kristensen, M., Lorenzen, J., Astrup, A., et al. (2015). Metabolomics investigation to shed light on cheese as a possible piece in the French paradox puzzle. *Journal of Agricultural and Food Chemistry, 63*, 2830–2839.
Zunabovic, M., Domig, K. J., Pichler, I., & Kneifel, W. (2012). Monitoring transmission routes of *Listeria* spp. in smoked salmon production with repetitive element sequence-based PCR techniques. *Journal of Food Protection, 75*, 504–511.