UNIVERSITA DEGLI STUDI DI CATANIA

FACOLTA DI AGRARIA

Dipartimento di Orto Floro Arboricoltura e Tecnologie Alimentari
Sezione Tecnologie Alimentari

Dottorato in Scienze e Tecnologie Alimentari- XXIV Ciclo
PhD Research in Food Science Techonology- XXIV Cycle

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Caratterizzazione Genotipica e Fenotipica di Ceppi di *L. rhamnosus* di Origine Umana e Alimentare

Genomic and Phenotypic Characterization of *L. rhamnosus* Strains from Human and Food Origin

Doctoral thesis

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Triennium 2008-2011

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List of original publications

This PhD thesis is referred on the following publications

- Cinzia L. Randazzo, **Angela Ribbera**, Iole Pitino, Cinzia Caggia. **Pecorino Crotonese cheese: Study of bacterial population and flavour compounds**. *Food Microbiology* 27 (2010) 363-374.

- Cinzia L. Randazzo, **Angela Ribbera**, Iole Pitino, Flora V. Romeo, Cinzia Caggia. **Diversity of bacterial population of table olives assessed by PCR-DGGE analysis**. *Food Microbiology* 32 (2012) 87-96.

- François P. Douillard, **Angela Ribbera**, Hanna M. Järvinen, Ravi Kant, Taija E. Pietilä , Cinzia Randazzo, Lars Paulin, Pia K. Laine, Cinzia Caggia, Ingemar von Ossowski, Justus Reunanen, Reetta Satokari, Seppo Salminen, Airi Palva1 & Willem M. de Vos. **Comparative Genomic and Functional Analysis of *Lactobacillus casei* and *rhamnosus* Strains Marketed as Probiotics**. Submitted to *Applied and Environmental Microbiology* (2012).

- François P. Douillard, **Angela Ribbera**, Ravi Kant, Hanna M. Järvinen, Marcel Messing, Taija E. Pietilä, Cinzia L. Randazzo, Lars Paulin, Pia Laine, Jarmo Ritari, Cinzia Caggia, Reetta Satokari, Stan Brouns, Tanja Lähteinen, Justus Reunanen, Ingemar von Ossowski, Airi Palva, Willem M. de Vos. **Comparative genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains from human and food origin**. Submitted to *Genome Biology* (2012).

Abstract

The aim of the present study was to investigate the dual role of Lactic Acid Bacteria in food fermentation processes and in health-promoting effects on human host.

In the first part of this PhD thesis will be described the role of spontaneous lactic acid microflora developed during fermentation of two traditional fermented foods: “Pecorino Crotonese” cheese and Table Olives, cultivar “Nocellara Etnea” and “Geracese”. The employment of culture-independent methods such as Denaturing Gradient Gel Electrophoresis (DGGE), gives the opportunity to trace the evolution of lactic acid microflora during the ripening of these traditional foods. Next to this, physical and chemical analyses have been integrated in order to highlight the main changes in food matrix and relate them as a consequence of microbial fermentation.

The second part of this study will focus on the analysis of probiotic factors of *L. rhamnosus*, a well-known and documented beneficial microorganism. Considering that *L. rhamnosus* is a multi niche species, several strains have been recovered from different sources such as fermented foods (Pecorino Crotonese cheese) and clinical samples and compared at genotypic and phenotypic level with the further aim to understand the evolution and the ecological versatility of this species. Moreover, *L. rhamnosus* strain GG and *L. casei* are two species widely marketed as probiotics, and a comparative analysis of some health-promoting traits will be provided in order to highlight differences in their claimed beneficial effects.

Part I

LAB and Food Fermentation

Introduction

Fermentation of food and beverage is one of the oldest ways of food processing. In the past the term fermentation was referred to anaerobic energy metabolism reflecting the foaming occurring during the preparation of beverages like wine and beer. Presently fermentation of foods is defined a bioprocessing using microorganisms and their enzyme to achieve desirable quality characteristics e.g. attractiveness, utility and functionality of fermented food (1). The **attractiveness** refers to the exterior, texture, odour and taste of food, all relevant aspects detectable by the sense and satisfying the consumer. **Utility** feature includes the reduction of bulk volume, shortening the cooking time, lengthening the shelf-life and improvement of nutrient retention. **Functionality** of fermented food relates to food safety, digestibility, probiotic effects and other beneficial impacts on the health and physiology of the consumer (30). This latter example is probably the major reason why people experienced in a good manner with fermented food and continue to cherish them. Fermentation bioprocessing requires several fundamental elements: composition of the food, microorganisms and water. In addiction physical, thermal and biological operations are required in organized and sequential way in order to make it a process (85). Many foods are fermented naturally that means without the use of specific microbial starter. In such cases the endogenous microflora on the ingredients will be responsible of the main changes occurring during fermentation and provide specific properties to the product. This simple technique does not allow prediction or standardization of product quality and safety although some bioprocessing of traditional foods (mainly dairy and meat products) have been improved thanks to the knowledge's of microbial metabolisms (94). For large scale and standardized fermentations, the employment of defined microbial starters is fundamental. In such settings the ingredients will be pre treated in order to reduce the contaminant microflora and then inoculated with selected/activated pure cultures of starter microorganisms (47). In this case the bioprocessing will be a unit operation visualized in flow diagrams representing the manufacturing process. Among the microorganisms all groups, i.e. bacteria, yeasts and moulds, are encountered as functional microorganisms in food fermentations, in particular the non taxonomic group of Lactic Acid Bacteria is the most widely distributed in home scale and industrial processing of fermented dairy, meat, vegetable and cereal products (59). The main contribution in bioprocessing is the conversion of available carbon sources in lactic acid with resulting acidification of raw matrix, which is considered a critical parameter in food preservation. The metabolism of lactic acid bacteria improves sensorial properties of food matrix because their enzymatic activities such as glycolysis, lipolysis and proteolysis with resulting production of desirable volatile flavour compounds (71). Additional advantages deriving from the lactic acid bacteria metabolism is the production of a broad-spectrum antimicrobial compounds, i.e. bacteriocins that prevent food spoilage. For instance nisin produced by certain strains of

Lactococcus lactis, a starter widely used in dairy industry, has antimicrobial activity against *Bacillus*, *Clostridium, Listeria* and *Staphylococcus* (15). Two direct consequences derives from the employment of selected antimicrobial-producing lactic acid bacteria: the prolongation of shelf life and the reduced addition of chemicals additives to the final product. Finally, fermentative metabolism of selected lactic acid bacteria contributes to human health in term of bioavailability increasing the absorption of essential nutrients, producing antioxidants, vitamins or other nutraceuticals as low-calorie sugars (25). In the latter case, lactic acid bacteria have been defined as 'cell factories' because the possibility of engineering them metabolically and a requirement to reach this task is an extensive knowledge of the physiology and genetics of these microorganisms that is greatly expanded with the advent of genomic era (24).

In the following paragraphs lactic acid bacteria will be described in their role of starter culture in bioprocessing with particular emphasis on the main categories of fermented foods and the molecular methods employed for their characterization in artisanal products.

Lactic Acid Bacteria: Classification and Identification

Lactic acid bacteria (LAB) belong to the *Firmicutes* phylum, *Bacilli* class and *Lactobacillales* order. They constitute a group of Gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as major end product during carbohydrates fermentation (53). Historically LAB are associated with habitats rich in nutrients, such as food matrices and mucosal surfaces of mammals. The first systematic classification of LAB has been done in 1919 by Orla-Jensen that followed as criteria of grouping the morphology, mode of glucose fermentation, range of sugar utilization and growth at certain temperatures (**Table 1**) (76). As result of such phenotypical clustering LAB were comprised in the following genera: *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Major revisions in the taxonomy of LAB were published in Bergey's Manual in 1986 introducing the genera *Enterococcus*, *Lactococcus*, *Vagococcus*, *Carnobacterium*, *Tetragenococcus*, *Weissella* and *Oenococcus* (92). Since 1990 alternative methods to phenotypical and biochemical characterization have been developed, leading to the identification of new LAB at genus, species and subspecies level (80). Specifically, automatic DNA sequencing technology has allowed direct sequencing of 16S rRNA gene and the related targeted probes have been used for identification of lactococci (55), enterococci (9), lactobacilli from different niches (43), carnobacteria (10) from meat and differentiate vagococci from other LAB (109). However other molecular typing methods have been developed based on the rRNA gene such as the restriction

fragment polymorphism (RFLP) that appeared to be useful for species and subspecies recognition (41).

	Rods		Cocci							
Growth Condition	*Carnobacterium*	*Lactobacillus*	*Aerococcus*	*Enterococcus*	*Lactococcus Vagococcus*	*Leuconostoc Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*	*Weissella*
CO_2 from glucose	–	±	–	–	–	+	–	–	–	+
Growth at 10°C	+	±	+	+	+	+	±	–	+	+
Growth at 45°C	–	±	–	+	–	–	±	±	–	–
Growth in 6.5% ofNaCl	ND	±	+	+	–	±	±	–	+	±
Growth in 18% of NaCl	–	–	–	–	–	–	–	–	+	–
Growth at pH 4.4	ND	±	–	+	±	±	+	–	–	±
Growth at pH 9.6	–	–	+	+	–	–	–	–	+	–
Lactic acid	L	D,L,DL	L	L	L	D	L,DL	L	L	D,DL

Table 1. Phenotypic/biochemical characterization of LAB. Symbols +,- and ± refer to growth conditions of LAB genera listed in the table.

PCR technique still remain the most powerful tool for classification and identification purposes because it is possible amplify a gene or part of it from a limited amount of cells (and therefore DNA) for subsequent sequencing (55). A number of fingerprinting techniques based on PCR have been developed such as randomly amplified polymorphic DNA (RAPD) (108) that has been shown effective for distinguishing *Lactobacillus acidophilus* group and discriminating *L. plantarum* at strain level (51, 106). Another fingerprinting PCR-based method with similarities to RAPD is REP-PCR, which exploits conserved repetitive DNA sequences in bacterial genome and has higher reproducibility than RAPD (106). Other genotypic fingerprint methods are based on restriction endonuclease cleaving on the chromosomal DNA. The large generated fragments are then resolved by pulse field gel electrophoresis (PFGE) that is considered the gold standard in classifying strains because its high discriminatory power (99). Further technique that has proven to be useful in LAB classification is soluble protein patterns (101, 103). The methodology resolves in polyacrylamide gel electrophoresis the whole bacterial cell proteins and the resulting patterns is analysed statistically (103). A dataset of digitalized and normalized patterns from a large number of LAB has been constructed and the similarity clusters clearly correlate with results based on genetic data, i.e. rRNA sequences (80). The method can be used to assign a particular strain to a species when the

pattern is compared with those in the database. In several studies the genetic methods described above have been compared in classifying LAB and results suggest that all methodologies can be considered complement each other. However thorough identification and classification in bacterial systematics, it is still recommended to apply a polyphasic approach that take into account several phenotypic, biochemical and genotypic methods (100).

Industrial Use of LAB in Food Bioprocessing

A starter culture can be defined as a microbial preparation of a large numbers of cells of at least one microorganism to be added to a raw material in order to produce a fermented food by accelerating and steering its fermentation process. LAB have a central role in these processes and long and safe history (GRAS) of application of that as they cause a rapid acidification of raw material through the production of lactic acid from carbohydrate metabolism (12). In addition other metabolites result from their proteolytic system with production of amino acids that are precursors of flavour compounds (**Figure 1**)(110). In the past food fermentation was carried out from microflora naturally present in raw material and quality of end product was dependent on the microbial load and spectrum on substrata processed (94).

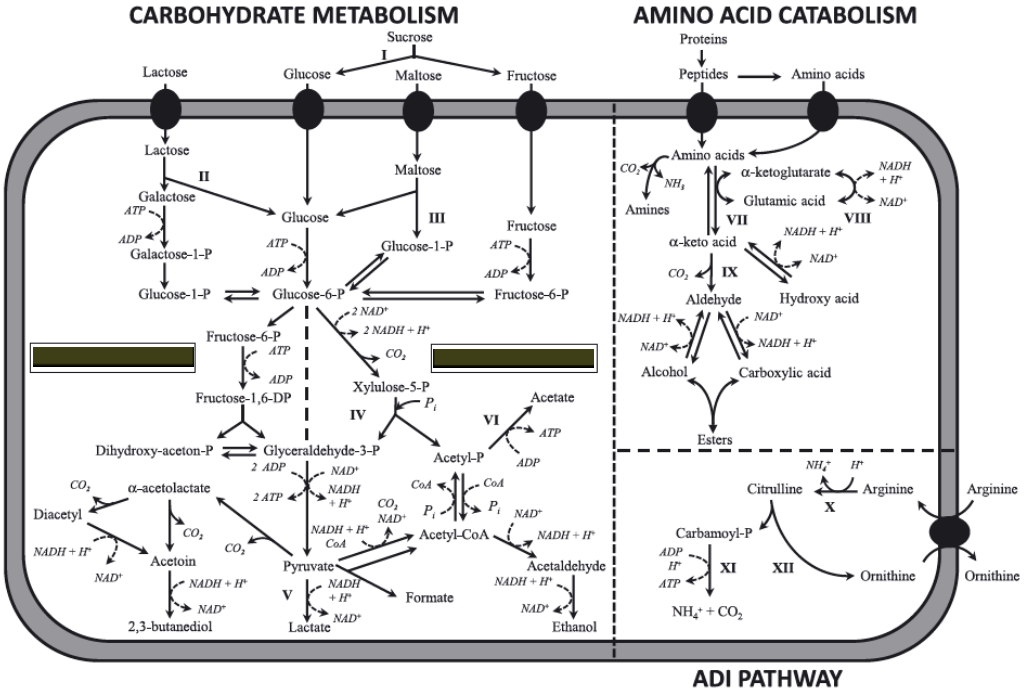

Figure 1. Major active pathways in LAB. Left panel: carbohydrate metabolism. Upper right panel: conversion of amino acids initiated by transamination in LAB. Lower right panel: arginine deiminase pathway.

Nowadays fermented food and beverage production represent a cheap and reliable method of preservation in less developed countries whereas in Western countries the large-scale production of fermented foods has became an important branch of food industry (47). The direct addiction of selected starter cultures to raw materials has been a breakthrough in processing of fermented foods, resulting in high degree of control over the fermentation process and standardization the end product. Strains with the proper physiological and metabolic features were isolated from natural habitats or from fermented products (75). Originally the initial selection of commercial starter cultures did not occur in a rational way and was mainly based on acidification and phage resistant properties. Moreover industrial starter cultures were propagated daily and probably this led to a shifting of the ecosystem resulting in a disappearance of certain strains. In addiction some important metabolites produced by LAB are plasmid-encoded and a daily propagation has increased the probability to loose genetic material due to the adaptation to the food matrix (12). A direct consequence of that was the reduced biodiversity of commercial strains and thus limited product diversity. In order to cope with this problem since the last decade a countertrend led to focus again in the natural ecosystem present in traditional fermented food, especially for those microflora named non-starter lactic acid bacteria (NSLAB), which develop in the product during maturation as a secondary flora together with coagulase-negative staphylococci (CNS) and filamentous fungi (33, 98). Pure culture isolates from complex ecosystem of traditional fermented foods exhibit a diversity of metabolic activity that strongly differ from the ones of comparable strains used as industrial bulk starters (54). These include differences in growth rate, adaptation to the substrate, antimicrobial activity, flavour aroma and quality attributes. In addition they are more dependent on their own biosynthetic capacities than industrial strains and harbour more amino acid converting enzymes that play a key role in flavour formation (6). Thus food industry is interested in isolation and characterization of wild type strains from traditional fermented products in order to use them as starter cultures in industrial fermentation process. In such way a product diversity and biodiversity of commercial starter is again regained (26).

Microbial characterization of LAB community in Fermented Foods

The most common approach to investigate the microbial community of interest in traditional fermented foods is the employment of culture dependent-techniques (96). These methodologies are based on microorganisms' growth in selective media and their subsequent identification at genus, species and strain level by the employment of molecular methods (see *Lactic Acid Bacteria: Classification and Identification* paragraph). However the study of biodiversity and the characterization of dominant microflora responsible for the peculiarity of traditional products employ culture-independent methods with the further aim to trace their evolution over space and

time in food ecosystem (52). At the same time the employment of this new molecular tools can be useful for monitoring quality and safety parameters in food production especially referring to the presence of hazardous microorganisms responsible for food born diseases (63). Compared to traditional culturing, these methods aim to obtain a picture of a microbial population without the need to isolate and culture its single components and are based on the examination of the total microbial DNA (or RNA) derived from mixed microbial population (36). Most of the culture independent techniques are based on PCR that since its introduction in the mid-1980 has become a fundamental tool to develop microbial community fingerprinting methods (**Table 2**).

Culture independent method	Taxonomic Resolution	Application	Example of Food matrix Investigation
PCR-DGGE/TGGE	Community members-genus and species level	Fingerprinting and population dynamics	Dairy, meat and cereal products
SSCP	Community members-genus and species level	Mutation analysis, fingerprinting and population dynamics	Cheeses and raw milk
T-RFLP	Community and population members-genus, species and strain level	Fingerprinting and dynamics between and with population	Milk and yoghurt
LH-PCR	Community members-genus and species level	Fingerprinting and population dynamics	Dairy starter, yogurt, cheeses, maize ensiling
PCR-ARDRA	Community members-species level	Microbial diversity within communities of isolated microorganisms	n/a
RISA	Particular community members-species group level	Fingerprinting and population dynamics	Sausages
FISH	Community members-species level	Detection of viable cells within communities, temporal and spatial distribution of microbes within ecosystems	Dairy products,
Multiplex FISH	Community members-species level	Similar to FISH, simultaneous investigation of complex communities	n/a

Table 2. Examples of culture-independent techniques widely used for food community investigation.

Although most of these PCR methodologies are generally based on the amplification of only variable regions or the totality of the 16S rRNA genes, amplified fragments can also derive from total RNA extracted from food and amplified by reverse transcriptase-PCR (RT-PCR) (91). Since active bacteria have a higher number of ribosomes than dead cells, the use of RNA instead of DNA highlights the metabolically active populations present in the ecosystem (107). PCR methods are rapid, easy to use, inexpensive and reproducible. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-temperature gradient gel electrophoresis (PCR-TGGE) were introduced more that 10 years ago in environmental microbiology and are now routinely used in may laboratories worldwide as molecular methods to study population composition and dynamics in food-associated microbial communities (73). There two techniques consist of the amplification of

the genes encoding 16S rRNA from the matrix containing different bacterial populations, followed by the separation of the DNA fragments. Separation is based on the decrease of electrophoretic mobility of PCR amplified, partially melted, double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (PCR-DGGE) or a linear temperature gradient (PCR-TGGE)(31). Molecules with different sequences may have different melting behaviour and stop to migrate at different position along the gel. The PCR-DGGE/TGGE generated patterns could provide a preliminary ecological view of predominant species increasing or decreasing in complex microbial communities by observing appearance or disappearance of species amplicons in the denaturing gel (74). PCR-DGGE have been applied to several fields of food microbiology for instance for the identification of microorganisms isolated from foods, the evaluation of microbial diversity during food fermentations and the assessment of the microbial and commercial food quality (35, 40, 64, 82, 83). Although PCR-DGGE/TGGE methods are reliable, reproducible and rapid, their limitation is that the community fingerprints they generate do not directly translate into taxonomic information. Thus the necessity of sequence the PCR-DGGE/TGGE bands and the following comparison of the nucleotide sequence with the available databases. Single-strand conformation polymorphism (SSCP)-PCR analysis detects sequence variations between different DNA fragments, which are usually PCR-amplified from variable regions of the 16S rRNA gene (89). This technique is essentially based on the sequence-dependent differential intra-molecular folding of single strand DNA, which alters the migration speed of the molecules. SSPC requires uniform, low temperature, non-denaturing electrophoresis to maintain single-stranded DNA secondary structure (102). The discriminatory ability of SSCP-PCR analysis depends on the position of the sequence variations in the gene studied. Similarly to PCR-DGGE/TGGE analyses, SSCP-PCR provides community fingerprints, which cannot be phylogenetically assigned. Terminal-Restriction Fragment Length Polymorphism (T-RFLP) is another PCR-based technique for profiling microbial community (77). Marker genes are amplified with fluorescently labelled primers, followed by restriction digestion, separation and detection on automated sequencer. Only labelled terminal restriction fragments (TRFs) are detected and their length heterogeneity indicates the complexity of the community visualized by an electropherogram. An internal size standard, labelled with a different fluorescent dye, allows precise length assignment with single-based pair resolution. With the 16S rRNA gene as target obtained TRFs can be compared to the sequence database of Ribosomal Databases Project allowing predictions of the microorganisms present in the analysed sample (18). Because one restriction enzyme often does not provide a sufficient resolution, multiple restriction enzymes can be used increasing the specificity and the reliability of the assay (68). Similarly to T-RFLP, Length Heterogeneity-PCR (LH-PCR) distinguishes different microorganisms basing on natural variation in the length of 16S rRNA gene sequences (95). In LH-PCR, a

fluorescently labelled oligonucleotides is used as forward primer; it is coupled with an unlabelled reverse primer to amplify hypervariable regions of the 16s rRNA gene, which are located at the 5'-end of the bacterial gene. Labelled fragments are resolved by capillary electrophoresis and detected by laser-induced fluorescence with an automated gene sequencer. As for T-RFLP, relationship between the size of amplicons obtained and gene phylogeny are predictable by comparison with previously published sequences of bacterial species (58). Amplified ribosomal DNA restriction analysis (ARDRA) is a relatively simple PCR-base fingerprinting technique based on the digestion of amplified ribosomal community DNA followed by gel electrophoresis that can be used for microbial identification or comparison of microbial communities and dynamics (72). In contrast to T-RFLP, all digested fragments are detected increasing the level of resolution. However single restriction enzyme does not provide sufficient resolution and multiple restriction enzymes have to be used either separately or in combination to obtain the desired resolution (52). Another drawback of this method is the limited staining sensitivity in gels resulting in the suppression of bands from less abundant community members or in a loss of phylogenetic information. As a consequence this molecular technique is advised to be used for less complex microbial community. Ribosomal intergenic spacer analysis (RISA) requires PCR amplification of total bacterial community DNA of the intergenic region between the 16S and 23S ribosomal genes (39). This intergenic spacer region displays significantly more heterogeneity in length and nucleotide sequence than the flanking region 16S and 23S ribosomal genes. In RISA size differences are exploited for subtyping of bacterial strains or in cases where fingerprinting of ribosomal sequence does not provide sufficient resolution. After gel electrophoresis of the PCR products, a complex community specific banding pattern is generated, with each band corresponding to at least one microorganism in the original community (8). The lack of sensitivity associated with this gel-based method led to development of automated RISA in which the original steps of DNA extraction and PCR are the identical to RISA, except for fluorescently labelled primer is used in the PCR (32). The electrophoresis resolution is performed on an automated system with laser detection of fluorescent DNA fragments. In order to increase and standardize the reproducibility different primers set can be used to examine a particular taxonomic group or species rather than the entire community (13). The PCR-based methods aforementioned have been applied for in polyphasic studies to monitor the microbial dynamics of food ecosystems (40). By combining different methods it is possible profile time-dependent specific shifts in the composition of complex food microflora, evaluate and quantify non-cultivable food populations and monitor the metabolically active microbial groups. However the aforementioned methods do not give exhaustive answers to cell physiology, cell-to-cell interactions and quantify non-cultivable or non-dominant species. Thus in situ methods have been introduced in order to identify and quantify cultivable and non-cultivable cells in minimally disrupted samples (3). The

fluorescence in situ hybridization (FISH) with rRNA targeted oligonucleotides fluorescent probes have been developed over the last decades aiming to visualized the temporal and spatial distribution of microbes in several ecosystems, included food matrices, revealing the morphology of the targeted microorganisms and how abundant they are in a given environment (7, 19, 22). Improvement of FISH has regarded the utilization at the same time of several probes carrying different fluorescent dyes with the simultaneous investigation of complex biofilms (97). In food microbiology the trend of molecular ecological studies is only getting started. In general the choice of an appropriate techniques to study microbial community depends on the aim of the research, the complexity of the community and the required resolution and sensitivity level. However when it comes to routinely monitoring a certain ecosystem on pre-defined characteristics, fingerprinting techniques such T-RFLP, DGGE, TGGE and SSCP produce a rough view on the microbial community composition and provide relevant data for subsequent in depth analysis. Moreover, in combination with sequencing or clone library analysis a more detailed profile can be obtained, allowing the design of DNA arrays and/or real time PCR assays. Nevertheless, the use of molecular techniques does not have to exclude traditional microbial culture methods as they can be used in combination to acquire more accurate and comprehensive results.

Fermented Products and Associated LAB

The variety of fermented foods produced is enormous, only dairy products count more then 1000 products, thus in the following paragraphs will be described the employment of LAB for the production of the main economically relevant categories of fermented foods (**Table 3**).

Category of fermented product	Type of Fermented product	Lactic acid Bacteria
Dairy Products	Hard cheeses without eyes	*Lc. lactis* subsp. *lactic*, *Lc. lactis* subsp. *cremoris*
	Cheeses with small eyes	*Lc. lactis* subsp. *lactic*, *Lc. lactis* subsp. *lactic biovar diacetylactis*, *Lc. lactis* subsp. *cremoris*, *Ln. mesenteroides* subsp. *cremoris*
	Swiss and Italian-type cheese	*L. delbrueckii* subsp. *lactis*, *L. helveticus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, *S thermophilus*
	Butter and buttermilk	*Lc. lactis* subsp. *lactic*, *Lc. lactis* subsp. *lactic biovar diacetylactis*, *Lc. lactis* subsp. *cremoris*, *Ln. mesenteroides* subsp. *cremoris*
	Yoghurt	*L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
	Fermented milk	*L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. johnsonii*
	Kefir	*L. kefir*, *L. kefiranofacies*, *L. brevis*
Fermented Meats	Fermented Sausages (Europe)	*L. sakei*, *L. curvatus*
	Fermented Sausages (USA)	*P. acidilactici*, *P. pentosaceus*
Fermented Vegetables	Sauerkraut	*Ln. mesenteroides*, *L. plantarum*, *P. acidilactici*
	Fermented Olives	*Ln. mesenteroides*, *L. pentosus*, *L. plantarum*
	Pickles	*Ln. mesenteroides*, *L. brevis*, *P. cerevisiae*, *L. plantarum*
Fermented Cereals	sourdough	*L. sanfransiscensis*, *L. farciminis*, *L. fermentum*, *L. brevis*, *L. plantarum*, *L. amylovorus*, *L. reuteri*, *L. pontis*, *L. panis*, *L. alimentarius*, *W. cibaria*
Alcoholic Beverages	Wine (malolactic fermentation)	*O. oeni*
	Rice wine	*L. sakei*

Table 3. Common LAB associated to fermented foods and beverages.

LAB in Fermented Dairy Products

Dairy starter cultures are actively growing culture of LAB that are added to the milk to target the fermentation process (49, 86). They are used in the production of a variety of dairy products, including cheeses, fermented milks and cream butter (49). LAB species employed as starters cultures belong to genera *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Streptococcus*. Starters used in dairy products can be divided into mesophilic and thermophilic species according to the optimum growth temperature (98). Mesophilic cultures grow in temperatures of 10-40°C, with the optimum around 30°C and the most used thermophilic LAB species are *Lc*. *lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, *Lc*. *lactic* subsp. *cremoris*, *Ln*. *mesenteroides* subsp. *cremoris*, *Ln*. *mesenteroides* subsp. *lactis*. Thermophilic cultures have an optimum growth temperature of about 42°C and the most used species are *S. thermophilus*, *L. delbrueckii* and *L. helveticus*. Usually mesophilic starters are used in the production of cheese varieties, fermented milk and ripened cream butter, while the thermophilic ones are employed for yogurt and cheese varieties production with high cooking temperature (5). All starter cultures available today are derived from **natural starters** (or artisanal) of undefined composition, i.e. containing an undefined mixture of different strains and/or species, and are still widely used in Europe and South America (17, 98). However for many dairy products, mainly cheeses, natural starters have been replaced by commercial **mixed-strain starters** (MSS) derived from the best natural starters and reproduced under controlled condition by specialized institutions. Both categories are called traditional starters and are opposed to **defined-strains starters** (DSS), composed of one or more strains, which were first used in New Zealand for cheddar cheese making in 1930s (62). Because of their optimized, highly reproducible, performance and their high phage resistance, DSS have replaced traditional starters in the production of many cheese varieties, including some PDO European varieties (16). **Table 4** summarizes the most common strains composing the traditional and DSS starters. The aforementioned categories of starter cultures constitute the primary starters, i.e. are involved mainly in the rapid acidification of milk because the production of lactic acid deriving from homo or hetero fermentative metabolism of lactose (78). Further feature deriving from the metabolism of primary starters is the production of antimicrobial compounds (excluding lactic acid and carbon dioxide) such as hydrogen peroxide and bacteriocins (69). A further relevant technological contribute deriving from LAB metabolism is the production of exopolysaccharides (EPSs) which contribute to the texture, stability, mouth-feel and taste perception of some dairy products (65). EPS-producing strains of *S. thermophilus* and *L. delbrueckii* species show a clear advantage when used in the manufacture of yogurt because syneresis and graininess are reduced whereas texture and viscosity are enhanced (61, 79). Although starter LAB cultures are responsible for initiating the milk bioprocessing, a second group called

secondary starters play a dominant role in dairy ripened products being responsible for the final features of fermented food (86).

Starter	Species	Lactose Fermentation	Metabolic Product	Fermented Products
Mesophilic	*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*	Homofermentative	L(+)-lactate	Cheddar, Cottage, Feta, Edam, Gouda, Camembert cheeses
	Lc. lactis subsp. *cremoris*	Homofermentative	L(+)-lactate	Viili
	Lc. lactis subsp. *lactis biovar diacetylactis*	Homofermentative	L(+)-lactate, diacetyl	Gouda, Edam, Cheddar, buttermilk, Nordic milks
	Ln. mesenteroides subsp. cremoris	Heterofermentative	D(+)-lactate, diacetyl, ethanol, C02	Cheedar, Buttermilk, Sour, Cream, Viili
	L. fermentum, *L. kefiranofaciens*, *L. casei*, *L. plantarum*, *L. curvatus*	Heterofermentative	D,L-lactate	Yogurt, Kefir, NSLAB in long ripened cheeses
Thermophilic	*S. thermophilus*	Homofermentative	L(+)-lactate, acetaldehyde, diacetyl	Yogurt, Gruyere,Emmental, Grana
	L. delbrueckii subsp. *bulgaricus*	Homofermentative	D(-)-lactate, acetaldehyde, diacetyl	Mozzarella
	L. acidophilus, *L. helveticus*	Homofermentative	D,L-lactate	Acidophilus milk, Gruyere, Emmental

Table 4. Common starter LAB used in dairy products.

Non-starter LAB (NSLAB) together with propionibacteria, coryneforms, staphylococci, yeasts and moulds are usually desirable contaminants of milk because they contribute to flavour formation by forming small peptides, amino acids and free fatty acids precursor of aromatic compounds (98). Usually NSLAB consist of a wide variety of strains that vary during the ripening time of dairy products, and their composition depends on the primary starters used for manufacturing. Strains belonging to the genus *Lactobacillus* (*L. casei*, *L. paracasei*, *L. plantarum*, *L. pentosus*, *L. curvatus*, *L. buchneri*, *L. brevis*), *Pediococcus* (*P. acidilactici*, *P. pentosaceus*) and *Enterococcus* (*E. durans*, *E. faecalis*, *E. faecium*) constitute the common non-starter lactic acid microflora recovered (93). However each dairy ripened fermented product harbours a specific group of NSLAB whose diversity depend on geographical and technological factors that underlie the product diversity (23). The wide diversity of NSLAB predominantly derives from artisanal cheeses produced mainly in the South of Europe for which the complexity of microbial communities has been identified and characterized by the employment of culture-independent methods (81).

LAB in Vegetable-based Products

Fermentation of plant material is an ancient preservation method whose origins are traced back to Asia (11). The most common products in Europe and United States are sauerkraut, cucumber and olives that are mainly manufactured in the Mediterranean region (67). The sequence of natural fermentation and storage of vegetables has been divided into four stages: initiation of fermentation, primary fermentation, secondary fermentation and post fermentation (34). Since the original amount

of LAB in vegetables is at most 1%, the aerobic organisms and the facultatively anaerobic enterobacteria are active at the beginning of fermentation. The primary fermentation is dominated by LAB and yeast. Their growth rate depends on several factors, including the physical and chemical properties of the vegetable and the environment (27). Secondary and post fermentation stages are caused by spoilage bacteria, yeast and moulds that use the residual sugars or acids as substrata. LAB that dominate in spontaneous vegetable fermentation belongs to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weisella* (**Table 5**) and usually they do not reduce nitrate that accumulate naturally in vegetables acting as a source of N-nitroso compounds (67). Starter cultures applied in vegetable fermentation must possess appropriate and specific characteristics depending on the properties of the fermented commodity. For instance selected strains of *L. plantarum*, *P. acidilactici* and *Ln. mesenteroides* cause a uniform fermentation and rapid acidification, good flavour formation and repression of yeasts growth when applied for bioprocessing of vegetable mixture of carrot, beet and cabbage (50).

LAB species	Fermented raw material
L. plantarum	Tomatoes, marrows, carrots, cucumbers, eggplants, red-beets, capers
L. pentosus	Capers, eggplants, cucumbers
L. fermentum	French beans, red beets, capers, eggplants
L. curvatus	Peppers
L. brevis	Tomatoes, capers, eggplants, cabbages, cucumbers
L. paraplantarum	Cabbages, capers
Ln. mesenteroides	White cabbages, carrots, peppers, cucumbers, eggplants
W. solii	Carrots
W. confusa, W. cibaria,	Peppers, tomatoes
P. pentosaceous	French beans, tomatoes, cucumbers, capers, cabbages.

Table 5. Fermented vegetable products and associated LAB

However the cultures currently employed present some limitations in their fermentative performances such as (i) the reduce rapid acidification, (ii) poor flavour development, (iii) low metabolic flexibility. Consequently high performing commercial starters are quite rare. Selection of starter strains should prefers autochthonous vegetable species and the main criteria of selection should be based on (i) technological, (ii) sensory and (iii) nutritional properties (67). Environmental

adaptation of presumptive starters is the primary requisite which affects all the other potential metabolic features (27). Concentration of fermentable carbohydrates, buffering capacity, pH and the presence of inhibitory compounds are the main environmental factors affecting the growth and acidification of lactic acid bacteria. Tolerance of phenols is indispensable to grow on some plant materials where such compounds are particularly abundant (87, 88). *L. plantarum*, together with close related *L. paraplantarum* and *L. pentosus*, seems to be a good candidate to carry out vegetable fermentations because posses a broad portfolio of enzymes such as β-glucosidase, p-cumaric acid decarboxylase, that have the capacity to degrade oleuropein and hydroxycinnamic acid derivates (57, 88). In addition several strains, isolated from various vegetable materials, can ferment the main carbon sources of the ecosystem, i.e. fructose, gentibiose, glucose, mannitol, mannose, methyl-glucoside and sucrose avoiding the growth of yeasts that usually metabolized the residue carbohydrates after lactic fermentation (28). Successfully employment of autochthonous *L. plantarum* starter was used for tomato juice fermentation in which high levels of ascorbic acid, total antioxidant activity and viscosity were higher during the storage when compared to bioprocessing of commercial *L. plantarum* strain (29). Among fermented vegetables, table olives are being extensively studied in bioprocessing because this product category is becoming economically relevant thus the necessity to characterize the microflora and standardize the process (14).

Species	Olive cultivar	Processing Method
L. plantarum	Green olive (Spain)	Treated
L. plantarum, L. paracasei, L. pentosus, Ln. pentosaceous	Galega green olive (Portugal)	Natural
L. plantarum, Enterococcus spp.	Green olive (Spain)	Treated
L. plantarum, Pediococcus spp.	Edincik and Gemlik black olive (Turkey)	Natural
L. plantarum. L. brevis, Lc. lactis, Ln. mesenteroides, P. damnosus	Green olive (Turkey)	Natural
Lc. lactis, L. plantarum, E. faecalis	Green olive (Algeria)	Natural
L. casei, L. plantarum, L. brevis, E. faecium	Green olive (Italy)	Natural
L. casei, L. rhamnosus, L. paracasei, L. plantarum, Lc. lactis, E. faecalis, E. faecium, E. durans	Sigoise green olive (Algeria)	Natural
L. plantarum, Ln. mesenteroides, P. pentosaceous,	Lecino black olive (Italy)	Natural
L. pentosus	Conservolea black olive (Greece)	Natural
L. plantarum, L. brevis, L. veridesens, L. curvatus, L. casei, Ln. mesenteroides	Jijelia black olive (Algeria)	Natural
L. coryniformis, L. paracasei, L. plantarum, L. pentosus, L. rhamnosus, L. brevis, L. casei, Lc. lactis, W. cibaria, E. italicus	Bella di Cerignola green olive (Italy)	Treated
L. pentosus, L. coryniformis	Nocellara del Belice green olive (Italy)	Treated

Table 6. Species of LAB identified in natural and treated table olives.

Although the lactic acid microflora of olives depends on cultivar and processing methods (natural or treated), *L. plantarum*, *L. pentosus* and *P. pentosaceus* are the main species recovered together with *L. casei* group species and *Ln. mesenteroides* (**Table 6**) (46). The employment of starter LAB in olive fermentation should promote high acidification rate reducing the risk of spoilage, tolerance to brine salinity that usually range between 4 and 15% (w/v) and resistance to polyphenol content that inhibit the growth of most LAB (20). Further criterion of culture LAB starters is the selection of bacteriocinogenic strains effective against *Propionibacterium*, *Clostridium* and *Listeria* genera. Bacteriocin production is conditioned by sodium chloride, initial pH and temperature (48). High bacteriocin gene expression is usually related to bacterial growth and the subsequent action is optimal when fermentation conditions are achieved. Interest in using bacteriocin producers as starters is considerable because it is an important parameter to increase the quality and the safety of fermented table olives (46).

LAB in Fermented Meat Products

Fermented dry sausages are non-heated meat products, mostly made from a mixture of pork meat and fat. During the grinding, additional ingredients such as glucose, lactose, salt nitrate and/or nitrite, ascorbate and spices are added (4). The final mixture is then stuffed into casing and hung vertically to be fermented at temperature comprised between 20°C and 30°C at high relative humidity. During fermentation the pH decrease due to the acidification of LAB, making the meat proteins coagulate resulting in the slice stability, firmness and cohesiveness found in the final product (60). Today the modern meat industry aims to ensure high quality, reduce variability and enhance organoleptic characteristics in sausage production. Starter cultures have been selected during the last 50 years reducing fermentation times, ensuring low residual nitrate and nitrite contents in the end product (44). Most of the commercially available starters are LAB mixed with staphylococci and micrococci strains that possess nitrate reductase activity. These starters can be divide in two categories: first generation starter preparations, which contains LAB responsible of a rapid acidification, such as *L. plantarum*, *P. acidilactici* and *P. pentosaceus* and second generation starters preparation containing LAB originating from meat and thus specially adapted to the ecology of meat fermentation (45). *L. sakei* and *L. curvatus* are most used species as second generation starters because predominant in naturally fermented sausages during the ripening, inhibiting the spontaneous lactic microflora responsible of excessive acidification and gas production causing at the end pungent off-flavour and holes of different sizes, respectively (45). Proteolytic activity is quite weak in meat LAB starters, however *L. sakei* has a superior competitiveness because posses arginine deiminase (ADI) pathway responsible for amino acid degradation (84). Therefore *L. plantarum* and *L. curvatus* contribute to the hydrolysis of sarcoplasmatic proteins and the

subsequent decomposition of peptides into amino acid that can be metabolized from CNS and moulds such as *Penicillium* contributing to the flavour formation (37). An additional result deriving from meat LAB metabolism is the improved safety by inactivation of food born-pathogens by the employment of bacteriocinogenic starter strain (15).

LAB in Fermented Cereal-based Products

Cereals are in general a good medium for microbial fermentation. They are rich in polysaccharides, which can be used as source of carbon energy by microbes in fermentation (90). The major polysaccharide in cereals is the starch, which became available to microbial fermentation after grain soaking and milling. In particular maltose, the energy microbial source highly present in dough, is metabolized via maltose phosphorylase pathway and the pentose phosphate shunt in heterofermentative LAB species (66). **Table 7** shows the main cereal-based foods resulting from LAB fermentation.

Operation	Material mixed with water	Principal purpose	Side effect or simultaneous reaction	Examples of a typical products
Soaking of grains prior to wet-milling	Whole grains	Softtening of grain endosperm	Lactic acid fermentation, control of undesired microorganisms	Ogi,agidi,koko,mawè
Slurrying or dough making after wet-milling	Wet starchy material from wet-milling	Separation of hulls etc from the starchy endosperm	Flavour production, control of undesired organisms	Ogi, agidi, kenkey, mawè
Slurrying after dry milling	Coarse meal from dry-milling	Separation of hulls etc.. from the starchy endosperm	Lactic acid fermentation, control of undesired microorganisms	Kiese, flummery
Dough for bread making	Flour	Aeration of dough	Acidification, flavour production, increase of mold-free time, control of α-amylase activity	Rye Sourdough bread
Malting	Malting barley	Germination, release of nutrients, increase of alfa-amylase activity	Control of undesired microorganisms	Barley malt
Brewing	Malted or unmalted cereal	Ethanol and flavour production	Acidification, flavour production	Traditional beers, lambic beer
Cooking a gruel	Maize or sorghum meal	Lactic acid, flavour production	Control of undesired microorganisms	Mageu

Table 7. Some functions of LAB in cereal-based fermented foods.

Sourdough is the most popular product belonging to this category of fermented foods and the fermenting LAB originate from the kernels and their initial count is around 10^2-10^3 cfu/gr. During fermentation process they rich up to 10^7 cfu/gr and together with yeast contribute to the rising process (21). A typical stable sourdough is a micro-ecological system that contains one to three major species of lactobacilli and yeast that establish a symbiotic relationship. In bread-making the heterofermentative lactobacilli play a major role and the acetic acid formed is essential to bread flavour and shelf life although obligate and facultative homofermentative lactobacilli can be found (56). Lactobacilli isolated from several kinds of sourdoughs are *L. acidophilus*, *L. casei*, *L. brevis*

and *L. fermentum* although *L. sanfranciscensis* and *L. pontis* are the main obligatory heterofermentative LAB recovered (2). In some specific fermenting processes high temperature are required in the first step in order to control the contaminant microflora and *L. delbrueckii* is used as starter because thermophilic species (70). Although proteolysis by LAB is limited in sourdough, acidification through carbohydrate breakdown activates endogenous proteases that release peptides and amino acids that can be taken up by other endogenous LAB and converted in precursors of flavour-active compounds (38). Specific and dedicated pathways may be involved, for instance *L. sanfranciscensis* and *L. reuteri* have found to display glutaminase activity that convert glutamine into glutamic acid, improving their acid tolerance (105). The conversion of arginine into ornithine via ADI pathway by *L. pontis*, *L. fermentum*, *L. brevis* and *L. sakei* is responsible of the characteristic flavour of baked wheat bread crust (42). Moreover production of ESPs by fermentative metabolism of lactobacilli affects water absorption of dough, rheology and machinability, stability during frozen storage and loaf volume as alternative to expensive addition of plant polysaccharides (65). However sourdough is employed for products that require a bakery process in order to be ready to eat. Some traditional cereal-based foods contain live LAB during the entire shelf life of the products, for instance the beers made in traditional ways deriving from the alcoholic and lactic fermentation (90). Species belonging to genus *Lactobacillus* and *Pediococcus* are used for lambic beer made from barley and wheat, a speciality of Belgium in which the fermentation process is very long and requires at least two years (104). Other traditional beverage that use as main ingredient maize to produce non-alcoholic beer employ strains of *L. plantarum*, *L. delbrueckii* and *L. bulgaricus* to increase the amylolytic activity (90). LAB are utilized in the production of cereal-based products in many ways and their fermentation contribute beneficially to the processing and to quality of the end products in term of flavour, keeping properties, safety and overall the attractiveness of the products, thus scientific research in combination with technological development aim to reproduce in industrial scale traditional products that are more appreciated from the consumers.

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Aim of the study

The task of the present study was to investigate the evolution of LAB microflora in two traditional fermented foods by the using of DGGE aiming to prove the versatility of this culture independent technique in tracing biodiversity and population dynamics. Molecular analysis of total fermenting microbial population has been integrated with traditional cultures methods and physiochemical analysis in order to monitor changes in food matrices due to fermentative metabolism of spontaneous microflora, starter cultures employed and the occurrence of pathogens bacteria.

Food Microbiology 27 (2010) 363–374

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Pecorino Crotonese cheese: Study of bacterial population and flavour compounds

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ARTICLE INFO

Article history:
Received 30 June 2009
Received in revised form
4 November 2009
Accepted 7 November 2009
Available online 11 November 2009

Keywords:
Bacterial population
PCR-DGGE
Flavour formation
Artisanal cheese
Polyphasic approach

ABSTRACT

The diversity and dynamics of the dominant bacterial population during the manufacture and the ripening of two artisanal Pecorino Crotonese cheeses, provided by different farms, were investigated by the combination of culture-dependent and -independent approaches. Three hundred and thirty-three strains were isolated from selective culture media, clustered using Restriction Fragment Length Polymorphism and were identified by 16S rRNA gene sequencing. The results indicate a decrease in biodiversity during ripening, revealing the presence of *Lactococcus lactis* and *Streptococcus thermophilus* species in the curd and in aged cheese samples and the occurrence of several lactobacilli throughout cheese ripening, with the dominance of *Lactobacillus rhamnosus* species. Bacterial dynamics determined by Denaturant Gradient Gel Electrophoresis provided a more precise description of the distribution of bacteria, highlighting differences in the bacterial community among cheese samples, and allowed to detect *Lactobacillus plantarum*, *Lactobacillus buchneri* and *Leuconostoc mesenteroides* species, which were not isolated. Moreover, the concentration of flavour compounds produced throughout cheese ripening was investigated and related to lactic acid bacteria presence. Fifty-seven compounds were identified in the volatile fraction of Pecorino Crotonese cheeses by Gas Chromatography–Mass Spectrometry. Esters, alcohols and free fatty acids were the most abundant compounds, while aldehydes and hydrocarbons were present at low levels.

1. Introduction

The occurrence of bacterial population, especially of lactic acid bacteria (LAB), during manufacture and ripening of most cheese varieties is already well documented (Beresford et al., 2001; Wouters et al., 2002). The origin of microorganisms may vary, entering from milk and/or with other ingredients used in cheese-making, or adventitiously from the environment, and LAB are considered the microrganisms mainly involved in flavour formation of cheese variety (Fox et al., 1996). It is noteworthy that the development of unique flavours in cheese is the result of complex reactions, e.g. glycolysis, lypolysis and proteolysis, mainly due to enzymes from milk, rennet and microorganisms (Fox, 1989). The proteolysis is undoubtedly the most important biochemical process for flavour and texture properties of semi-hard and hard cheese types. Proteolytic enzymes from LAB play an important role in the degradation of casein and peptides leading to the production of free amino acids, which are rapidly converted into specific volatile compounds by nonstarter lactic acid bacteria (NSLAB) as well as by lactococci (Ayad et al., 2000; Amarita et al., 2001; Kieronczyk et al., 2003). Several studies have demonstrated the occurrence of LAB species in several Italian cheeses like Canestrato Pugliese (Aquilanti et al., 2006), Parmigiano Reggiano (Gala et al., 2008), Pecorino (De Angelis et al., 2001; Randazzo et al., 2006, 2008), Ragusano (Randazzo et al., 2002), Raschera and Castelmagno (Dolci et al., 2008a,b), Provola dei Nebrodi (Cronin et al., 2007), Fontina (Giannino et al., 2009); in several Spanish artisanal starter-free cheese types (Oneca et al., 2003; Sánchez et al., 2006; Abriouel et al., 2008; Martín-Platero et al., 2008), and in French cheeses (Duthoit et al., 2003; Callon et al., 2004). Up to now no information is available on the composition of the bacterial population and on flavour formation throughout cheese manufacture and ripening of Pecorino Crotonese (PC) cheese.

Pecorino Crotonese is an artisanal cheese manufactured on a small scale by farmers, following traditional practices, in a well-defined area of Southern Calabria (Italy). It is produced from raw ewes' milk with the addition of kid rennet paste. According to a traditional protocol, the use of starter culture is not allowed and the acidification is due to the autochthonous lactic acid bacteria (LAB). Hence, the quality of raw milk, the environmental conditions and the traditional manufacture play a major role in determining the characteristics of this artisanal PC cheese and have a clear effect on the microbial population. Characterizing cheese microbial population may contribute to understand the ecological processes that drive microbial interaction in cheese and their technological relevance.

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0740-0020/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2009.11.010

At present, a wide range of molecular approaches is available to study bacterial community in cheese, including culture-dependent and -independent techniques.

Culture-dependent techniques, based on cultivation followed by phenotypic and molecular identification, are known to be laborious and time-consuming to monitor population dynamics and may over- or underestimate the microbial diversity (Randazzo et al., 2002; Ercolini et al., 2004). In the last decade, the profiling of bacterial populations became more precise with the application of molecular techniques based on the direct detection of DNA and RNA in microbial ecosystems. The application of the Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S rRNA gene to study microbial communities and to monitor their dynamics during manufacture and ripening of artisanal cheeses has recently been reviewed (Randazzo et al., 2009).

The aim of the present study was to evaluate bacterial population of PC cheese through a combination of culture-dependent and -independent approaches, in order to obtain a complete description of the dominant species involved during manufacture and ripening and to assess their contribution to flavour formation by Solid Phase Micro Extraction (SPME) and Gas Chromatography–Mass Spectrometry (GC–MS) analyses.

2. Materials and methods

2.1. Cheese-making procedure and sampling

The Pecorino Crotonese cheese-making procedure is already documented (Gardini et al., 2006). Two kinds of PC cheese are currently produced: semi-ripened (60-days-old) and ripened (up to 2 years-old) one. The cheese samples used in this study were collected from two farmers (A and B) from two different areas of Crotone (Calabria, Italy), and the cheeses were chosen based on their high quality properties and collected in two-consecutive weeks. Curd, semi-ripened (60 days) and ripened cheese (120 days) samples were aseptically taken, in duplicate, during cheese manufacture and ripening, and they were subjected to bacteriological analysis within 6 h or stored at −80 °C.

2.2. LAB reference strains and culture conditions

The LAB reference strains *Enterococcus faecalis* DSM #20468^T, *Enterococcus faecium* DSM #20478^T, *Enterococcus hirae* DSM #20160^T, *Lactobacillus brevis* DSM #20054^T, *Lactobacillus buchneri* DSM #20057^T, *Lactobacillus delbrueckii* subsp. *lactis* DSM #20072^T, *Lactobacillus fermentum* DSM #20052^T, *Lactobacillus helveticus* DSM #20075^T, *Lactobacillus paracasei* subsp. *paracasei* DSM #5622^T, *Lactobacillus pentosus* DSM #20314^T, *Lactobacillus plantarum* DSM #20246^T, *L. plantarum* subsp. *plantarum* DSM #20174^T, *Lactobacillus rhamnosus* DSM #20021^T, *L. rhamnosus* GG, *Lactococcus lactis* subsp. *lactis* DSM #20481^T, *Streptococcus thermophilus* DSM #20617^T used in this study came from the Deutsche Sammlung von Mikroorganismen und Zelkulturen (Braunschweig, Germany). Dairy wild strains *Lactobacillus curvatus* RC23, *Lactobacillus paraplantarum* F3, *L. lactis* subsp. *cremoris* LC1, and *Leuconostoc mesenteroides* CR310 were taken from DOFATA microbial collection. Lactococci, enterococci and streptococci were cultivated on LM17 medium, M17 medium (Oxoid, Basingtoke, United Kingdom) supplemented with 5 g l^{-1} of lactose, and *Leuconostocs* on MRS agar (Oxoid). Incubation was performed at 32 °C and 42 °C for 24–48 h for mesophilic and thermophilic bacteria, respectively, under anaerobic conditions using an Anaerogen kit (Oxoid, Milano, Italy).

2.3. Enumeration and isolation of LAB

Samples (10 g) of curd were taken directly during cheese-making and two diametrically opposed samples (10 g) of semi-ripened and ripened cheeses (60 and 120 days, respectively) including either the cheese core or surface were cut up, ground in a sterile food mill, pooled, serially diluted in 90 ml sterile physiological solution (0.9% NaCl), and homogenized with a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for approximately 5 min. The samples were analyzed by plating appropriate ten-fold dilutions onto the following media: PCA (Oxoid,) for mesophilic aerobic bacteria; MRS (Oxoid), acidified to pH 5.4 with hydrochloric acid (HCl, 1 M); Rogosa agar (Oxoid) for mesophilic and thermophilic lactobacilli; LM17 medium containing cycloheximide (Fluka Chimica, Milan, Italy) (100 μg l^{-1} added after sterilization) for *Lactococcus* and *Streptococcus*; MSE agar (Biolife, Milan, Italy) for *Leuconostoc*; and KAA agar base (Kanamycin Aesculin Azide, Oxoid), containing Kanamycin Selective Supplement (Oxoid), for enterococci. Plates containing MRS and KAA agar media were incubated under anaerobic conditions using an Aerogen kit at 37 °C for 48–72 h LM17 plates were incubated at 32 °C and 42 °C for 24–48 h for mesophilic and thermophilic cocci, respectively and plates containing MSE medium were incubated at 30 °C for 48–72 h.

2.4. Phenotypic identification of LAB isolates

To characterize the bacterial population, a representative number of colonies was randomly picked from various agar plates and each colony was purified by streaking three times. All isolates were subsequently cultured on LM17 and MRS agar and finally stored at −20 °C in the same media, containing 20% glycerol, before being subjected to physiological, technological and genotypic identification.

All isolates were characterized by determining their Gram reaction, their catalase activity, spore formation, and ability to grow in MRS broth at 10 °C and 45 °C in stationary tubes. Cell morphology was observed with a phase contrast microscope.

2.5. Technological characterization of LAB strains

The technological properties of LAB strains studied in the present work were: the ability to acidify and to coagulate both ewes' and skim milk powder (Oxoid). The ability of strains to acidify ewes' milk and sterile skim milk powder (reconstituted at 100 g l^{-1}) containing 0.1% of Yeast Extract (Oxoid), was determined using a pH-meter (Eutech Instruments, XSPH 510, Nijkerk, The Netherlands), after 8 h of incubation at 30 °C. The coagulating activity was evaluated by the appearance of visual coagulum on the inner site of glass tube containing both ewes' and powder milk.

2.6. DNA extraction from bacterial strains and from dairy samples

Genomic DNA from bacterial isolates and reference strains was extracted from 6 ml of overnight grown cultures as described by Gala et al. (2008). Total DNA extraction from dairy samples was performed according to the protocol previously described by Randazzo et al. (2002).

2.7. PCR amplification

PCR amplification was performed in a 50 μl volume using a GenAmp PCR System 9700 (Perkin–Elmer, Foster City, CA, USA). The reaction mixtures consisted of 1.25 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 50 μM dNTPs, 5 pmol primers each and 1 μl of properly diluted template DNA. The reaction mixture with no template DNA was used as a negative control. The universal primers 7-f and 1510-r (Lane, 1991) were used to amplify the 16S ribosomal RNA gene of the isolates and the reference strains. The cycling program was the following: initial denaturation of DNA for 5 min at 94 °C; 35 cycles

each consisting of 30 s at 94 °C, 30 s at 56 °C and 40 s at 68 °C; and extension of incomplete products for 7 min at 68 °C. PCR products were visualized by electrophoresis on a 1.2% (w/v) agarose gel containing ethidium bromide, and where necessary, they were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Universal primers F357 (5′-TACGGGAGGCAGCAG-3′) and R518 (5′-ATTACCGCGGCTGCTGG-3′) were used to amplify the V3 region of the bacterial 16S rRNA gene (Muyzer et al., 1993). A GC clamp (5′-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGG-3′) was linked to the first primer to obtain F357-GC.

To investigate the dominant bacterial communities by DGGE analysis PCR products were generated using PCR primers U968-GC and L1401-r to amplify the V6 to V8 region of eubacterial 16S rDNA (Nubel et al., 1996). The 40-nucleotide GC rich sequence at the 5′ end of primer U968-GC improves the detection of sequence variations of amplified DNA fragments by subsequent TGGE/DGGE (Muyzer et al., 1993). The samples were amplified in a Perkin–Elmer Applied Biosystem GenAmp PCR System 9700 (Foster City, CA, USA) programmed as follows: initial denaturation of DNA for 5 min at 94 °C; 35 cycles each consisting of 30 s at 94 °C, 30 s at 56 °C and 40 s at 68 °C; and extension of incomplete products for 7 min at 68 °C.

The samples were amplified as described above and the PCR conditions were essentially those described by Muyzer et al. (1993).

2.8. Restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA genes

The RFLP analyses of the 16S rRNA gene PCR products of isolates and reference strains, obtained using the universal primers 7-f and 1510-r mentioned above, was performed by restriction enzymes *Hae*III, *Alu*I and *Msp*I (Gibco BRL, Praisley, UK), at 37 °C for 2 h, followed by electrophoresis of the products on a 2% (w/v) agarose gel in 1× TBE buffer (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA; pH 8.0) containing ethidium bromide.

2.9. Denaturant gradient gel electrophoresis (DGGE) analysis

DGGE analysis of PCR amplicons was performed on the Dcode System apparatus (BioRad, Hercules, CA), as previously described (Muyzer et al., 1993). Electrophoresis was performed in a 0.8 mm-thick polyacrylamide gel 8% [w/v], acrylamide:bisacrylamide [37.5:1] containing a urea plus formamide gradient from 30% to 60%, increasing in the direction of the electrophoresis run. Optimal separation was achieved with 40–60% urea-formamide denaturant gradient, increasing in the direction of electrophoresis. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. The gels were subjected to a constant voltage of 85 V and at temperature of 60 °C for 15 h in 0.5× TAE buffer. The DNA bands were visualized by silver staining and developed as previously described (Sanguinetti et al., 1994).

2.10. Cloning and sequencing of 16S rRNA gene in plasmid inserts

Clone libraries of the 16S rRNA gene amplicons from curd, fresh and ripened cheeses of farmer I and II were constructed. Amplicons derived from PCR of the 16S rRNA gene using primer pairs 7-f and 1510-r were purified and cloned in *Escherichia coli* JM109 using the pGEM-^{T} plasmid vector system (Promega, Madison, USA) in accordance with the manufacturer's instructions. Appropriate regions of the 16S rRNA gene in the cell lysates of the transformants were amplified using the primers pair U968-GC and L1401 and their mobility was compared to the rDNA-derived patterns of curd and cheese samples by DGGE (data not shown). The clones that produced a single DGGE amplicon with a melting position identical to that one of the dominant bands in the curd and cheese DNA patterns were selected for sequence analysis.

In order to identify the strains belonging to RFLP clusters which did not mach to any reference strain, pure cultures of strains were sequenced by Biodiversity s.p.a. (Brescia, Italy) company. To determine the closest known relatives of the isolates, partial 16S rRNA gene sequences were compared to those in the GenBank database (http://ncbi.nlm.nih.gov/BLAST/) and those of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) using the BLAST program (Altschul et al., 1997). Sequences with a percentage identity of 97% or greater were considered to belong to the same species.

2.11. Physico-chemical analyses

Chemical analysis such as pH, water activity (Aw), titrable acidity and chemical composition like total solids, proteins, salt, and fats of Pecorino Crotonese cheese samples were performed according to IDF Standards (1979, 1982, 1986a,b, 1989). Each sample was analyzed in triplicate.

2.12. Analysis of volatile compounds

The analysis of volatile compounds was carried out by GC–MS and sample were extracted using Solid Phase Micro Extraction (SPME) following the protocol described by Randazzo et al. (2008). The absorbed volatiles were then analyzed by GC–MS using a Hewlett–Packard 6890 gas-chromatograph equipped with a Hewlett–Packard 5973 quadruple mass selective spectrometer. The separation was achieved by a HP-5 fused-silica capillary column (30 m × 0.2 mm, film thickness 0.25 mm); the oven temperature program was the following: 35 °C for 3 min, 5 °C min^{-1} to 110 °C, then 10 °C min^{-1} to 240 °C and 240 °C for 10 min; the carrier gas flow was set to: 1.8 ml min^{-1}; the injector temperature was 250 °C; and the detector temperature 250 °C. The eluted compounds were identified by matching their mass spectra with those of the Wiley 175 library (Wiley & Sons, Inc., Germany), or those of the pure standard components and then confirmed by their GC retention times.

2.13. Statistical analysis

All experiments were performed in duplicate and the experimental data were reported as average value and provided with Standard Deviation. Statistical ANOVA ($P > 0.01$) and Duncan tests were performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA). Statistical ANOVA was carried out to evaluate the effect of the ripening on the bacterial growth on different media of samples provided from the 2 different farmers. Moreover, a two-way ANOVA was performed in order to compare the microbial loads of the two different samples at each sampling point.

Statistical treatment of SPME data was carried out using the SPSS 11.0 software package (SSPS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was applied to the data to determine the presence of significant differences among volatile compounds during ripening (Duncan's test, significant level $P < 0.05$).

3. Results

3.1. LAB diversity using plate counts

The mean of microbial counts and standard deviation obtained by classical enumeration of bacterial population present in the PC samples during cheese manufacture and ripening are shown in Table 1. Results of ANOVA analysis, performed on samples provided from the two different farmers, are shown in the same Table 1. Overall, almost all microbial groups showed a significant increase

Table 1
Mean log of lactic acid bacteria population using plating counts.

Samples	Microbial log counts (expressed as mean of cfu g^{-1}) and standard deviations (SD)						
	PCA Mesophilic aerobic bacteria	MRS Mesophilic lactobacilli	RA Thermophilic lactobacilli	LM17 at 32 °C Lactococci	LM17 at 45 °C Streptococci	MSE Leuconostoc	KAA Enterococci
Curd A	5.2 ± 0.30^{a}	4.35 ± 0.38^{a}	1.39 ± 0.00^{a}	6.61 ± 0.04^{a}	6.21 ± 0.30^{a}	5.06 ± 0.65^{a}	2.6 ± 0.00^{a}
60 days PC cheese A	7.88 ± 0.20^{b}	8.62 ± 0.52^{b}	8.23 ± 0.59^{b}	8.85 ± 0.06^{b}	8.16 ± 0.24^{b}	8.34 ± 0.78^{b}	7.54 ± 0.76^{b}
120 days PC cheese A	7.20 ± 0.08^{b}	7.94 ± 0.05^{b}	6.09 ± 0.55^{c}	7.29 ± 0.62^{a}	5.79 ± 0.04^{a}	8.10 ± 0.45^{b}	7.57 ± 0.16^{b}
Curd B	5.9 ± 0.40^{a}	4.20 ± 0.98^{a}	3.10 ± 0.09^{a}	4.70 ± 0.41^{a}	1.84 ± 0.64^{a}	3.66 ± 0.58^{a}	3.59 ± 0.55^{a}
60 days PC cheese B	7.88 ± 0.20^{b}	7.55 ± 0.01^{b}	7.63 ± 0.08^{b}	7.61 ± 0.04^{b}	5.53 ± 0.33^{b}	7.68 ± 0.01^{b}	7.40 ± 0.04^{b}
120 days PC cheese B	7.45 ± 0.27^{b}	7.50 ± 0.29^{b}	7.47 ± 0.24^{b}	7.54 ± 0.23^{b}	5.63 ± 0.18^{b}	7.59 ± 0.09^{b}	7.49 ± 0.26^{b}

Mean values of two independent samples taken throughout cheese manufacture and ripening; in the same column followed by different lowercase letters are significantly different ($P < 0.01$).

during the 60 days of ripening and a slight decrease up to 120 days. Regarding cheese supplied by farmer A, mesophilic aerobic bacteria showed a significant increase in the 60-days-old cheeses, maintaining a constant value up to the end of ripening. Mesophilic lactobacilli, present in curd sample (load of 10^4 cfu g^{-1}) increased significantly (to 10^8 cfu g^{-1}) in the 60 days cheese samples, and it maintained a similar concentration of 120 days of ripening. Thermophilic lactobacilli showed a significant increase up to the 60 days of ripening (from initial value of 10–10^8 cfu g^{-1}) and a significant decrease of 120 days of ripening, reading the value of 10^6 cfu g^{-1} (Table 1). Both mesophilic and thermophilic cocci showed a similar trend, with a significant increase at beginning of the ripening, and reaching final values similar to the initial ones. Leuconostoc and enterococci counts exhibited similar trend, with a significant increase at the 60 days of ripening, and keeping a constant value up to the end of ripening.

In the samples supplied by farmer B all microbial groups exhibited a significant increase in cheese after 60 days of ripening and kept a constant concentration in the 120-days-old cheese (Table 1).

3.2. Identification of bacterial isolates

Three hundred and thirty-three isolates were considered LAB based on their positive Gram reactions, nonmotility, absence of catalase activity and spore formation and rod or coccal shape (data not shown). Thirty-eight of the isolates produced gas from glucose, indicating a heterofermentative metabolism (Table 2). One hundred and four isolated grew at both 10 and 45 °C after incubation for 5 days and 48 h, respectively. Two hundreds and eighty-three of the isolated strains were mesophilic and grew at 10 °C but not at 45 °C. One hundred and twenty-eight isolated grew well only at 45 °C.

Amplification of the partial 16S rRNA gene and subsequent restriction analysis with endonuclease *Hae*III, *Alu*I and *Msp*I were performed and the different restriction profiles were compared to those obtained from type strains in order to aid identification at species level. The PCR-RFLP of the 16S rRNA gene analysis allowed grouping the strains into 8 clusters. Within the cluster I the 190 strains were ascribed to *L. rhamnosus/paracasei*. Twenty-eight strains from cluster II and 18 from cluster III were classified respectively as *E. faecalis* and *S. thermophilus*. Cluster IV and V included lactococcal strains belonging respectively to the species *Lactococcus lactic* subs. *cremoris* (1 strain) and *L. lactis* (31 strains). Sixty-five strains did not mach to any reference strain used for RFLP analysis considered in the present study; thus one for each representative cluster was submitted to 16S rRNA gene sequencing (Table 3). Thirty-four strains, ascribed to cluster VI, belonged to the *L. brevis* species, 26 isolates from cluster VII and 5 isolates from cluster VIII belonged to *L. mesenteroides* species.

The frequency of isolation of LAB species throughout manufacture and ripening of PC cheeses is reported in Fig. 1. *L. rhamnosus/pracasei* was the most dominant species (57%), followed by *L. brevis* (10.2%), *L. lactis* (9.3%), *L. mesenteroides* (9.3%), *E. faecalis* (8.4%), while *S. thermophilus* (5.4%) and *L. cremoris* (0.4%) were present at minor levels (Fig. 1).

While the highest biodiversity was observed in the curd samples, where at least 5 different LAB species were detected, throughout the whole ripening period the number of the species decreased. Moreover the distribution of the LAB species differed between curd and cheese samples, and cheeses provided by different farmers, as well. In detail, among LAB isolates, *E. faecalis* and *L. mesenteroides* species were dominant in the curd A, and their frequency was 35.3% and 26.5%, respectively. From the curd B the species *E. faecalis* was also isolated even if with a lower percentage than 15%. In aged cheese A *E. faecalis* was not detected while in cheese B it appeared only at low frequency (14.1%) at 120 days of ripening.

In contrast, *L. mesenteroides* species was detected in the 60 days ripened cheese and disappeared in the 120 days cheese.

L. lactis was the most frequent species isolated in the curd B, with 46.6% of frequency. *S. thermophilus* isolates, which were present only in curd A at 8.8% of frequency, decreased during the ripening of the cheese, while they appeared in the 60 and 120 days ripened cheese B with frequencies ranging from 13.6% to 5.6%, respectively. In aged cheese samples, *L. rhamnosus/paracasei* was the most frequent species isolates with percentages ranging from 81.5% to 79.2% in samples supplied by farmer A and from 72.7% to 56.3% in the samples provided by farmer B samples. Isolates belonging to *L. cremoris* species were constantly present in the curd A, while *L. brevis* species was both in the curd and in 60-days cheese provided by farmer A.

3.3. Technological characterization of the isolates

Results of technological properties of the 333 isolates are shown in Fig. 2. Thirty-seven strains isolated from curd A and 42 strains isolated from curd B, showed a different frequency of coagulant activity. In detail, within strains from curd A, only 11% showed good coagulant activity while 89% of the strains did not coagulate the milk. On the contrary, 66% of the strains from curd B, showed good coagulant activity and 12% and 31% of the strains registered a very poor or no activity, respectively.

A different trend was pointed out by strains isolated from 60 days cheese samples. In fact, 69% of the 61 isolates from cheese A showed good performance and only 31% did not coagulate the milk. Most of the isolates (98%) from 60 days cheese B did not coagulate the milk.

Most of the 123 strains isolated from 120-days-old cheeses A and B did not coagulate the milk (Fig. 2).

3.4. DGGE analysis of bacterial population and identification of the dominant species

DGGE fingerprinting of the total bacterial community of Pecorino Crotonese cheese using two different pairs of primers was carried out by amplifying both the V6 to V8 and the V3 regions of

Table 2
Phenotypic and molecular identification of strains isolated from Pecorino Crotonese cheese.

Isolates	Source	Medium	Morphology	Growth		CO_2 from glucose	RFLP profile & sequencing
				10 °C	45 °C		
A11, A13, A14, A31, A54, A61, A64, B45, B61, B63, B64, F44, F45, F53, F57, G56, G61	Curd	KAA	Cocci in pairs	+	–	–	*E. faecalis*
A12, F25	Curd	MRS	Rods	+	–	–	*L. rhamnosus*
A16, A25, B37	Curd	MRS	Rods	–	–	–	*L. rhamnosus*
A23, A27, A53, B11, B12 B13, B14, B16, B23, F16, F21, F22, F23, F24, F56, G21, G24, G25, G26	Curd		Cocci in pairs	+	–	+	*L. mesenteroides*
A42, B31, B44	Curd		Cocci in pairs	–	–	–	*L. mesenteroides*
A51, A63, B57	Curd	M17	Cocci in pairs	–	+	–	*S. thermophilus*
B35	Curd	KAA	Cocci in pairs	–	–	–	*E. faecalis*
B46	Curd	M17	Cocci in pairs	+	–	+	*L. lactis* subsp. *cremoris*
B62, G27, G41, G42, G45, G51	Curd		Rods	+	+	–	*L. brevis*
F11, F13, F32, F35, F51, F54, F61, F62, F65, G11, G12, G13, G14, G31, G38 G64	Curd	M17	Cocci in pairs	+	–	–	*L. lactis*
F17, G44	Curd	MRS	Rods	+	+	–	*L. rhamnosus*
G53	Curd		Rods	+	–	–	*L. brevis*
A32, B30, B33, B55, G63	Curd	M17	Cocci in pairs	–	–	–	*L. lactis*
D13, D15, D16, D21, D22, D23, D24, D25, D26, D34, D51, D52, D54, D55, D61, D63, D65, E11, E12, E15, E16, E21, E22, E23, E44, E53, E55, D62, D64, D66, E13, E14, E24, E25, E26, E32, E41, E61, E62, E63, E65, E66, N11, N12, N13, N15, N16, N21, N22, N24, N25, N31, N32, N33, N34, N35, N41, N51, N52, N53, N54, N55, N56, N61, N62, N63, N64, N65, N66, P12, P13, P14, P15, P21, P22, P26, P31, P32, P33, P34, P35, P36, P51, P54, P61, P62, P63, P64, P66, P55, P65, P56	Cheese (60 days)	MRS	Rods	+	+	–	*L. rhamnosus*
D31, D33 D36, D42, E45	Cheese (60 days)	M17	Cocci in pairs	+	–	–	*L. lactis*
D32, D46	Cheese (60 days)		Cocci in pairs	–	–	–	*L. mesenteroides*
D35	Cheese (60 days)		Cocci in pairs	+	–	–	*L. mesenteroides*
D43	Cheese (60 days)		Rods	–	–	–	*L. brevis*
D44, E33, E34, E36, E42	Cheese (60 days)	MRS	Rods	–	–	–	*L. rhamnosus*
E31, N42, N43, N44, N46, P42, P43, P45, P46, P47	Cheese (60 days)	M17	Cocci in pairs	–	+	–	*S. thermophilus*
E43	Cheese (60 days)		Cocci in pairs Cocci in pairs	–	–	–	*L. mesenteroides*
E51	Cheese (60 days)		Cocci in pairs	+	–	+	*L. mesenteroides*
E52, P25	Cheese (60 days)	MRS	Rods	+	–	–	*L. rhamnosus*
N14	Cheese (60 days)		Rods	–	–	+	*L. brevis*
N23, N26, P24, P53	Cheese (60 days)		Rods	+	–	–	*L. brevis*
N45	Cheese (60 days)	MRS	Rods	–	+	+	*L. rhamnosus*
P16, P52	Cheese (60 days)		Rods	+	–	+	*L. brevis*
P23	Cheese (60 days)		Rods	+	+	+	*L. brevis*
P41	Cheese (60 days)		Rods	–	+	+	*L. brevis*
P44	Cheese (60 days)	MRS	Rods	–	+	–	*L. rhamnosus*
H11, H12, H13, H14, H15, H21, H22, H23, H24, H25, H26, H31, H32, H34, H51, H55, H61, H62, H63, H64, H65, L11, L12, L13, L14, L15, L16, L21, L22, L23, L24, L25, L26, L31, L32, L33, L54, L62, L63, L64, L65, Q12, Q13, Q14, Q15, Q16, Q21, Q22, Q23, Q24, Q25, Q32, Q35, Q41, Q52, Q53, Q61, Q62, Q63, Q65, R11, R13, R15, R16, R21, R22, R23, R26, R31, R32, R33, R34, R35, R36, R51, R61, R62, R63, R64, R65	Cheese (120 days)	MRS	Rods	+	+	–	*L. rhamnosus*
L51	Cheese (120 days)		Cocci in pairs	+	+	–	*L. mesenteroides*
H42, L45, R41, R42, R44	Cheese (120 days)	M17	Cocci in pairs	–	+	–	*S. thermophilus*
H44	Cheese (120 days)	M17	Cocci in pairs	–	+	–	*L. lactis*
H52	Cheese (120 days)		Cocci in pairs	–	+	–	*L. mesenteroides*
H53, L53	Cheese (120 days)		Cocci in pairs	+	+	+	*L. mesenteroides*

(*continued on next page*)

Table 2 (*continued*)

Isolates	Source	Medium	Morphology	Growth		CO_2 from glucose	RFLP profile & sequencing
				10 °C	45 °C		
L34, L44, L56, L66	Cheese (120 days)	M17	Cocci in pairs	+	–	–	*L. lactis*
Q11, Q26, Q31, Q33, R56, R66	Cheese (120 days)		Rods	+	–	–	*L. brevis*
Q34, Q36	Cheese (120 days)	MRS	Rods	+	–	–	*L. rhamnosus*
Q42, Q43, Q44, Q45, Q46, Q54, Q66, R45, R46, R52	Cheese (120 days)	KAA	Cocci in pairs	+	+	–	*E. faecalis*
Q51, Q55, Q56	Cheese (120 days)		Rods	–	–	+	*L. brevis*
Q64	Cheese (120 days)		Rods	–	–	–	*L. brevis*
R12, R14	Cheese (120 days)		Rods	+	+	+	*L. brevis*
R25, R53, R54, R55,	Cheese (120 days)		Rods	+	–	+	*L. brevis*
R43	Cheese (120 days)		Rods	–	+	–	*L. brevis*

the 16R rRNA gene. DGGE profiles of V3 hyper-variable amplicons of curd, 60 and 120 old cheese samples derived both from the two different farmers are shown in Fig. 3. The appearance and disappearance of amplicons in the DGGE patterns indicate important shifts in the microbial community structure. In general, the evolution of bacterial community throughout ripening process was reflected in the unstable DGGE profiles (Fig. 3). An increase in diversity was observed during ripening, with new bands appearing in samples from curd sample to 120-days-old cheese. DGGE profiles of curd and cheese samples (lanes I, II and III, Fig. 3) were generally different and typical for each farm, suggesting strong differences in the bacterial composition.

In order to identify the most dominant bands in the DGGE profiles, reference bacterial strains were chosen as ladder and used in this study to allow the comparison among gels (data not shown). In addition, clone libraries of the partial 16S rRNA gene amplicons from 60-days-old cheese samples were constructed in order to identify some of the dominant bands in the rDNA-derived patterns. In detail, DGGE profiles obtained from curd A (lane I, Fig. 3) showed the dominance of *L. lactis* subsp. *lactis* (band 1), which remained stable throughout ripening, and the presence of *L. brevis* (band 2) and *L. buchneri* (band 3), which were also revealed in cheese samples (lines II and III, Fig. 3). Interestingly, during ripening, new species, including *L. plantarum/pentosus* (band 4) *L. fermentum* (band 5), *L. mesenteroides* (band 6), *L. delbruechii* (band 7), *L. rhamnosus* (band 8), were detected. On the contrary, the most intense band emerged during manufacture and ripening process of samples B, corresponded to *S. thermophilus* (band 9, Fig. 3), which was not observed in the samples A. In addition to *S. thermophilus* in curd B (lane IV, Fig. 3) only band 11 was revealed, which corresponded to an uncultured bacterium (Table 3). Nevertheless, other *Lactobacillus* species were encountered in cheese B (lanes V and VI, Fig. 3), including *L. fermentum* (band 5), *L. mesenteroides* (band 6), *L. delbruechii* (band 7), *L. rhamnosus* (band 8). Two clones (9P and 10P) from the 60-days-old cheese resulted in sequences derived from species *L. lactis* subsp. *lactis* and corresponded to the band 10a and 10b. Three clones (21P, 29P, and 4D), which corresponded to uncultured bacterium (Table 3), showed identical position of bands 11, 12 and 13, respectively.

The same bacterial species were detected using primers U968-GC and L1401-r, excepted for *L. rhamnosus* and *L. paracasei* species, which showed identical profile such as *L. plantarum* and *L. pentosus* species (data not shown).

Table 3
Partial sequencing of the 16S rRNA gene of strains and clones from Pecorino Crotonese cheese samples.

Strain/clone	Closest sequence relative	% Identity	Accession number
F16	*Leuconostoc mesenteroides*	98%	EU419608.1
A42	*Leuconostoc mesenteroides*	97%	EU483104.1
R12	*Lactobacillus brevis*	97%	FJ405226.1
B30	*Lactococcus lactis* subsp. *lactis*	97%	FJ378886.1
9P	*Lactococcus lactis* subsp. *lactis*	97%	DQ173745.1
10P	*Lactococcus lactis* subsp. *lactis*	97%	FJ378885.1
21P	Uncultured bacterium clone	97%	EU464482.1
29P	Uncultured bacterium clone	98%	EF603471.1
4D	Uncultured bacterium clone	97%	DQ447546.1

3.5. Cheese physico-chemical characteristics

Table 4 shows the evolution of different chemical parameters throughout PC cheese ripening. In detail, total solid content was higher in cheese A than in cheese B, and showed a moderate increase throughout ripening in both cheeses (Table 4). In accordance with the increase of the total solids values in both cheese samples, the water activity decreased from initial values of 0.92 and 0.95 to final values of 0.85 and 0.92, in cheese A and B, respectively. A slight decrease of pH was observed in the first stage of ripening of cheese A, reaching a 5.05 final value in the 120-days-old cheese, while opposite trend (from 5.12 to 5.24) was registered by cheese B. Titratable acidity value was quite constant during the ripening of cheese A, while exhibited a higher drop (from 0.96 to 0.67) in cheese B (Table 4). The cheese B samples showed a higher protein content than cheese A at 60 and 120 days of ripening. The NaCl content was higher in cheese A than in cheese B and increased in both cheeses during ripening. The fat content was initially quite similar in both cheeses, but slightly decreased in cheese A and increased in cheese B during ripening.

3.6. Analysis of volatile compounds

Large differences of volatile compounds between cheese A and B samples, evaluated throughout ripening, were found. The revealed concentrations of hydrocarbons, terpenes, free fatty acids, alcohols, ketones, aldehydes, esters and sulphur compounds, determined both in the 60 and 120 days-old cheese samples, are shown in Table 5.

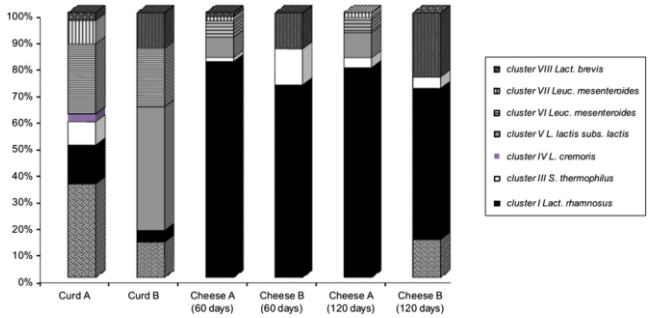

Fig. 1. Frequency of isolation of bacterial species throughout manufacture and ripening of artisanal Pecorino Crotonese cheeses.

3.7. Hydrocarbons

Five hydrocarbons were found in the volatile fraction of the PC cheese samples examined (Table 5). The highest concentration of hydrocarbons was detected in the cheese A samples but the compounds exhibited a significant decrease in both cheeses during ripening.

3.8. Terpenes

Among terpenes it is interesting to highlight that each of the seven identified compounds were found only in the 60 days cheese samples supplied by farmer A and none of them was detected in the ripened cheese samples of farmer B. Moreover, all terpenes concentration dramatically decreased throughout ripening.

3.9. Linear free fatty acids (FFAs)

Nine linear free fatty acids (FFAs) were identified in PC cheese, eight of which were simultaneously present in both cheeses, but exhibited different trend during ripening. In particular, butanoic and hexanoic acids, showed in cheese A the highest concentration, which was quite constant throughout the ripening process. In cheese B the amounts of the two acids increased significantly during ripening, reaching values approximally similar to those registered in cheese A samples. Their presence could be related to the rising of NSLAB throughout ripening. While the 3-methyl-butanoic acid appeared in both cheese samples only at 120 days, only dodecanoic acid was not detected in cheese samples provided by farmer B.

3.10. Alcohols

Ten alcohols were identified in the PC cheese samples, however only 4 of them were simultaneously present in both cheeses. Moreover, large differences between the cheeses were found throughout ripening (Table 5). In detail, the alcohol compound 2-methyl 2-buten-1-ol was detected only in 120 days cheese B samples while 1-butanol, 3-methyl-1-butanol, 1-pentanol, and 3-methyl-2-butanol compounds were present only in cheese A samples.

3.11. Ketones

Seven keton compounds were detected in ripened cheese samples. The highest keton concentrations were revealed in cheese B samples; in particular, 2-pentanone and 2-nonanone were the most abundant compounds, which showed the highest concentration in 60-days-old cheese and a significant decreasing in 120 days ripened cheese (Table 5).

3.12. Aldehydes

Only 2 aldehydes, nonanal and benzaldehyde, were detected and their concentrations decreased throughout ripening (Table 5). In detail, the nonanal was revealed at the same concentration, only

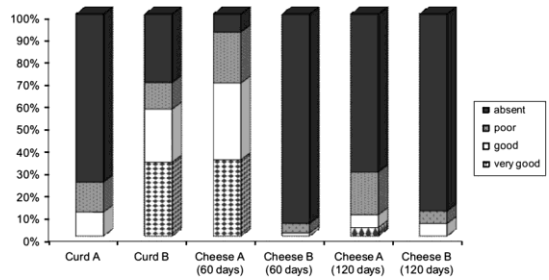

Fig. 2. Frequency of acidifying and coagulating activities of isolates during manufacture and ripening of Pecorino Crotonese cheeses.

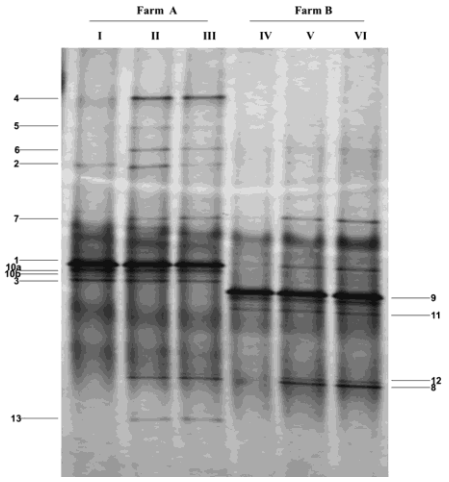

Fig. 3. Bacterial DGGE profile of PCR amplicons of the V3 region of the 16S rRNA gene of curd, and ripened Pecorino Crotonese cheeses, manufactured from two different farmers (A and B). Line I, curd A; line II, 60-days-old cheese A; lane III, 120-old cheese A; lane IV, curd BI; lane V, 60-days-old cheese B; lane VI, 120-old cheese B. Band 1, *L. lactis* subsp. *lactis*; band 2, *L. brevis*; band 3, *L. buchneri*; band 4, *L. plantarum/pentosus*; band 5, *L. fermentum*; band 6, *L. mesenteroides*; band 7, *L. delbruechii*; band 8, *L. rhamnosus*; band 9, *S. thermophilus*; band 10a and 10b, *L. lactis* subsp. *lactis*; bands 11, 12, and 13: uncultured bacteria.

in cheese A samples, while the benzaldehyde was found in both cheese samples showing a significant decrease during ripening.

3.13. Esters

Fifteen esters were identified in the volatile fraction of Crotonese cheese samples (Table 5). Among the detected esters, most of them were ethyl and methyl esters. In general the highest concentration of ester compounds was revealed in the cheese provided by farmer A. The ethyl butanoate and ethyl hexanoate were mostly present, showing a significant increase during the ripening of the PC cheese A and a significant decrease in cheese B. The highest amount of ester was butanic acid-ethyl ester which was registered in cheese A and exhibited a significant decreased during ripening.

Table 4
Physico-chemical characteristics of Pecorino Crotonese cheeses. Standard deviation values are in parentheses.

Parameters	Cheese A		Cheese B	
	60 days	120 days	60 days	120 days
Total solids[a]	72.05 (0.26)	75.50 (0.14)	67.00 (0.10)	68.08 (0.54)
Aw (Eq/L)	0.92 (0.02)	0.85 (0.02)	0.95 (0.01)	0.92 (0.02)
pH	5.14 (0.01)	5.05 (0.01)	5.12 (0.04)	5.24 (0.03)
Titratable acidity[b]	0.84 (0.01)	0.82 (0.01)	0.96 (0.03)	0.67 (0.03)
Proteins[c]	40.50 (0.30)	40.91 (0.40)	43.01 (0.40)	42.30 (0.35)
NaCl[c]	8.87 (0.01)	9.68 (0.01)	7.07 (0.01)	8.40 (0.01)
Fats[c]	46.80 (0.31)	46.12 (0.35)	46.20 (0.32)	47.43 (0.26)

[a] Expressed as g/100 g of cheese.
[b] Expressed as g of lactic acid/100 g of cheese.
[c] Expressed as g/100 of total solids.

3.14. Sulphur compounds

Only two sulphur compounds, such as carbon disulphide and methane-thiobis, were detected in all cheese samples, with the highest concentration in cheese B samples, where they decreased significantly during ripening.

4. Discussion and conclusion

Since no detailed studies focusing on the bacterial ecology during manufacture and ripening of PC cheese are available, in the present study a polyphasic approach, with culture-dependent and -independent methods, investigated the bacterial population and their dynamics in this product. Traditional plating results, using five different media, underlined the microbial concentrations in cheeses throughout manufacture and ripening. Both cheese samples, provided by different farmers, were characterized by a high level of all LAB groups, which showed a considerable increase at the beginning of ripening, as found in several ripened cheeses (Randazzo et al., 2002, 2006; Østlie et al., 2004; Martín-Platero et al., 2008). Although the high microbial counts registered in the LM17 medium, only a low number of strains belonging to *Lactococcus* spp. were detected in cheese B, and none in cheese A. Moreover, while the presumptive *Leuconostoc* number significantly increased on MSE medium throughout ripening of both cheese samples only a limited number of *Leuconostoc* isolates were detected. To a certain extend this can be explained by the lack of medium selectivity (Ercolini et al., 2001; Dasen et al., 2003).

In general, throughout the ripening period a decrease in biodiversity was observed and the distribution of LAB diversity differed between cheese samples and according to the stage of ripening. This could be explained by the change of cheese environment, and by parameters such as humidity, salt concentration, pH and total solid content. In details, the latter was higher in cheese A that in cheese B, probably due to the higher acidification rate resulting in more whey drainage, and showed a moderate increase throughout ripening in both cheeses probably due to the loss of water. Moreover, the higher concentration of total content in cheese A is probably due to the higher NaCl content, which could be correlated with the significant decrease of thermophilic lactobacilli, lactococci and streptococci during cheese ripening. Psoni et al. (2003) described how the high NaCl content was the main factor regulating microbial survival in Batzos cheese. In particular, the authors registered a significant decrease of lactococci during ripening due to the inhibitory effect of the high salt content, which may transform part of the lactococci population into viable but non-cultivable cells (VNC) or intact dead cells, which can be detected only by culture-independent approach, according to Casalta et al. (2009).

Following LAB population and its dynamics during manufacture and ripening by DGGE analysis, we obtained a complete understanding of the bacterial ecology of PC cheese, revealing the dominance, throughout making process and ripening, of *L. lactis* species in cheese A. This species corresponded in the DGGE profiles to a multiple bands, which may be related to the presence of multicopies of the 16S rRNA gene, according to previous studies (Bonetta et al., 2008; Casalta et al., 2009). Moreover, according to other reports (Florez and Mayo, 2006; Delbès et al., 2007; El-Baradei et al., 2008; Ercolini et al., 2008; Giannino et al., 2009), the amplification of the V3 region, within the 16S rRNA gene allowed us to distinguish between *L. rhamnosus* and *L. paracasei* species. Nevertheless, the related species *L. plantarum* and *L. pentosus* showed identical V3 sequences and cannot be distinguished. Ogier et al. (2002) also detected, using TGGE technique, species with identical V3 region and others which exhibited the same T_m (Murray et al., 1996) and thus migrate at the same position. Hence, more discriminating areas are needed to differentiate these strains.

Table 5
Volatile compounds detected in Pecorino Crotonese cheese samples during ripening.

Compounds	TR[a]	Cheese A		Cheese B		ANOVA
		60 days	120 days	60 days	120 days	
Hydrocarbons						
Hexane	1.3	0.055 ± 0.004^{b}	0.045 ± 0.011^{b}	0.376 ± 0.156^{a}	0.127 ± 0.018^{b}	*
1-6-Octadiene-3-7-dimethyl	5.72	0.846 ± 0.123^{a}	0.298 ± 0.004^{b}	0.633 ± 0.149^{a}	0.184 ± 0.014^{b}	**
3-Octene	2.27	0.136 ± 0.023^{c}	0.107 ± 0.012^{c}	3.190 ± 0.165^{a}	0.985 ± 0.014^{b}	***
Heptane	1.48	0.027 ± 0.014^{b}	0.014 ± 0.007^{b}	2.116 ± 0.219^{a}	t	***
Octane	1.92	0.018 ± 0.001^{a}	0.023 ± 0.005^{a}	t	t	**
Terpenes						
Limonene	9.98	2.352 ± 1.655^{a}	1.045 ± 0.105^{a}	–	–	NS
Sabinene	7.94	0.213 ± 0.074^{a}	–	–	–	**
Trans-carane	8.85	0.290 ± 0.011^{a}	–	–	–	***
α-Pinene	5.35	0.263 ± 0.009^{b}	0.558 ± 0.008^{a}	–	–	***
α-Terpinolene	12.59	0.101 ± 0.021^{a}	–	–	–	**
β-Pinene	7.45	0.203 ± 0.029^{a}	0.086 ± 0.009^{b}	–	–	***
γ-Terpinene	11.7	0.585 ± 0.169^{a}	0.528 ± 0.047^{a}	–	–	**
Linear fatty acids						
3-Methyl-butanoic acid	22.31	–	3.069 ± 0.049^{a}	–	3.637 ± 1.543^{a}	**
Butanoic acid	21.63	34.269 ± 1.135^{a}	34.282 ± 0.424^{a}	3.780 ± 2.386^{c}	29.429 ± 1.056^{b}	***
Decanoic acid	29.03	2.120 ± 0.070^{a}	1.520 ± 0.079^{b}	1.107 ± 0.176^{b}	1.350 ± 0.277^{b}	*
Dodecanoic acid	30.86	0.228 ± 0.008^{a}	0.135 ± 0.020^{b}	–	–	***
Hexadecanoic acid	35.65	0.924 ± 0.183^{a}	–	2.761 ± 2.607^{a}	1.314 ± 0.870^{a}	NS
Hexanoic acid	24.64	30.053 ± 1.841^{a}	28.475 ± 0.137^{a}	9.499 ± 3.025^{c}	23.394 ± 0.716^{b}	**
Octanoic acid	27	4.782 ± 0.250^{a}	3.835 ± 0.335^{b}	3.217 ± 0.013^{c}	4.714 ± 0.087^{a}	**
Pentanoic acid	22.38	0.828 ± 0.027^{b}	1.95 ± 0.073^{b}	4.759 ± 1.224^{a}	1.590 ± 0.824^{b}	*
Tetradecanoic acid	32.8	0.422 ± 0.230^{a}	0.521 ± 0.382^{a}	1.744 ± 1.449^{a}	0.747 ± 0.429^{a}	NS
Alcohols						
2-Buten-1-ol-2-methyl	4.6	–	–	–	0.346 ± 0.039^{a}	***
1-Butanol	9.16	0.215 ± 0.041^{a}	0.188 ± 0.011^{a}	–	–	**
1-Butanol-3-methyl	10.83	1.072 ± 0.129^{a}	0.882 ± 0.048^{a}	–	–	***
1-Hexanol-2-ethyl	18.55	–	–	1.422 ± 0.137^{a}	1.811 ± 1.124^{a}	NS
1-Pentanol	8.36	–	0.571 ± 0.042^{a}	–	–	***
1-Phenoxy-propanol	26.5	0.136 ± 0.005^{b}	0.138 ± 0.014^{b}	1.068 ± 0.098^{a}	1.529 ± 0.522^{a}	*
2-Butanol-3-methyl	8.15	1.227 ± 0.127^{a}	–	–	–	***
Heptanol	14.96	1.284 ± 0.239^{a}	0.586 ± 0.023^{b}	–	0.357 ± 0.011^{b}	**
Hexanol	14	0.159 ± 0.017^{bc}	0.118 ± 0.005^{c}	0.421 ± 0.099^{b}	0.892 ± 0.163^{a}	**
Phenol	26.1	0.213 ± 0.007^{a}	–	0.274 ± 0.065^{a}	0.412 ± 0.202^{a}	NS
Ketones						
2-Heptanone	10.03	1.479 ± 0.421^{a}	0.389 ± 0.003^{a}	–	–	**
2-Hexanone	7.07	–	–	0.975 ± 0.013^{a}	0.441 ± 0.015^{b}	***
2-Nonanone	15.88	0.724 ± 0.016^{c}	0.225 ± 0.004^{c}	13.058 ± 1.157^{a}	4.157 ± 1.017^{b}	***
2-Octanone	12.96	–	–	–	0.182 ± 0.010^{a}	***
2-Pentanone	4.46	0.346 ± 0.021^{c}	0.176 ± 0.009^{c}	14.391 ± 1.215^{a}	7.892 ± 0.974^{b}	***
2-Propanone	2.09	0.072 ± 0.005^{c}	0.068 ± 0.009^{c}	0.665 ± 0.025^{a}	0.391 ± 0.072^{b}	***
8-Nonen-2-one	17.33	–	–	0.427 ± 0.053^{a}	0.253 ± 0.052^{b}	**
Aldehydes						
Nonanal	16.04	0.121 ± 0.001^{a}	0.072 ± 0.045^{a}	–	–	*
Benzaldehyde	19.28	0.338 ± 0.049^{b}	–	0.940 ± 0.288^{a}	0.598 ± 0.089^{a,b}	**
Esters						
1-2-Benzene dicarboxylic acid diethyl ester	29.65	–	–	–	0.173 ± 0.013^{a}	***
1-Butanol-3-methyl acetate	8.31	0.343 ± 0.062^{a}	0.319 ± 0.015^{a}	–	–	**
2-Propenoic acid-6-methyl heptyl ester	12.06	–	–	0.682 ± 0.162^{a}	0.278 ± 0.029^{b}	**
Acetic acid-ethyl ester	2.84	0.658 ± 0.055^{a}	0.603 ± 0.048^{a}	0.630 ± 0.029^{a}	0.352 ± 0.099^{b}	*
Acetic acid propyl ester	12.47	–	1.101 ± 0.023^{a}	–	–	***
Butanoic acid 2-methyl propyl ester	9.29	–	0.095 ± 0.001^{a}	–	–	***
Butanoic acid hexyl ester	10.9	2.962 ± 4.039^{a}	0.116 ± 0.004^{a}	–	–	NS
butanoic acid-3-methyl-ethyl ester	6.8	0.060 ± 0.002^{b}	0.116 ± 0.010^{a}	–	–	***
Butanoic acid-ethyl ester	5.91	2.392 ± 0.396c	4.838 ± 0.324^{b}	14.659 ± 0.482^{a}	5.604 ± 0.510^{b}	***
Butanoic acid-methyl ester	4.67	0.109 ± 0.034^{b}	0.076 ± 0.001^{b}	0.965 ± 0.185^{a}	–	**
Decanoic acid-ethyl ester	21.35	–	–	0.287 ± 0.025^{a}	t	***
Hexanoic acid 2-methyl propyl ester	14.86	–	0.034 ± 0.009^{a}	–	–	**
Hexanoic acid-ethyl ester	11.49	6.729 ± 0.223^{c}	11.661 ± 0.076^{a}	8.888 ± 0.561^{b}	3.453 ± 0.239^{d}	***
Octanoic acid-ethyl ester	17.09	–	0.489 ± 0.070^{b}	1.058 ± 0.034^{a}	1.114 ± 0.221^{a}	**
Pentanoic acid-ethyl ester	8.59	0.082 ± 0.003^{b}	0.193 ± 0.005^{a}	t	t	**
Sulphur compounds						
Carbon disulfide	1.6	0.542 ± 0.172^{b}	0.657 ± 0.230^{b}	5.369 ± 2.001^{a}	2.053 ± 0.691^{b}	*
Methane-thiobis	1.69	0.018 ± 0.001^{c}	0.075 ± 0.006^{c}	0.626 ± 0.066^{a}	0.227 ± 0.017^{b}	***

[a] TR: retention time; NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Comparison bacterial community of cheese samples provided from different farmers, DGGE profiles highlighted differences, suggesting that each farmhouse or manufacturing facility may be characterized by an independent microbial population. Moreover, profiles revealed the appearance of new bands throughout ripening, indicating an increase in diversity, in discordance to conventional microbiological results. Bands corresponding to *L. lactis*, *L. buchneri*, *L. brevis*, and *L. plantarum/pentosus* dominated during the ripening of cheese A, while *S. thermophilus* was revealed only in samples B. These results confirmed that microbial population of any artisanal cheese is determined not only by the source of milk, but also by the manufacturing process, and by hygienic practices observed during cheese-making and ripening (Martín-Platero et al., 2008). Nevertheless, bands corresponding to *L. fermentum*, *L. rhamnosus*, *L. delbrueckii*, recurred in both ripened cheese samples, as reported for Spanish ewe's milk cheeses (Abriouel et al., 2008; Martín-Platero et al., 2008).

It is relevant that bands corresponding to *L. plantarum/pentosus*, *L. buchneri* and *L. mesenteroides* emerged very clearly in ripened cheese A, while they were not isolated from cheese samples. Plate counting of LAB could fail to detect some bacteria due to the inability of selective media to provide specific growth requirements and for the randomness in colony selection that could bring to the risk of a false identification (Jany and Barbier, 2008). In the present work the failure to recover these bacteria might be explained by their own inability to grow on solid media. They were either stressed or in a viable but non-cultivable state. On the other hand, *E. faecalis* was isolated from curd samples but not detected in the DGGE gel. This could be due to the efficacy of DNA extraction, which influences the amplification results and depends in turn on the bacterial species and food matrices (Abriouel et al., 2006; Pérez-Pulido et al., 2005). Moreover, species representing less that 1% of the total community would not be detected in the DGGE gel (Muyzer et al., 1993).

The abundance of LAB species in the final products, especially in those provided by farmer A, could be correlated to the high lactic acid production, to the slight decrease of the pH values and could explain the high concentration of volatile compounds (e.g. esters, FFAs, and alcohols). Esters were the principal volatile compounds in PC cheese, to which confer floral, fruity sweet notes. In particular the ethyl hexanoate plays an important role in the aroma profiles of many aged cheeses producing orange note (Curioni and Bosset, 2002). Esters are mainly produced by the enzymatic or chemical reaction of fatty acids with primary alcohols (Engels et al., 1997); moreover, they can also be formed by trans-esterification of partial glycerides (Holland, 2004). In the present work ethyl butanoate and ethyl hexanoate were the major ester compounds, showing different trend in cheese samples. In fact while in cheese A significantly increased during ripening in cheese B samples dramatically decreased. These results are in discordance to those reported by Dahl et al. (2000), that asserted that the increase of esters, throughout ripening, may be attributable to the parallel increase of the short- and medium chain of fatty acid concentration.

Regarding alcohols, they may be rapidly produced from aldehydes under the strong reducing conditions present in cheese (Molimard and Spinnler, 1996), or from other metabolic pathways, e.g. lactose metabolism and amino acid catabolism. These compounds generate fruity and nutty notes in some cheeses and when present at high levels, they are responsible for defects as revealed in Gouda and Cheddar cheeses (Engels et al., 1997). During the ripening of Pecorino Crotonese cheese, 10 alcohol compounds were detected, but their concentration was low. Among alcohol compounds, the 3-methyl-1-butanol is considered an important contributor to the cheese flavour and it is often associated to fruity taste (Moio et al., 1993). Moreover, aliphatic primary alcohols such as 1-butanol and 1-hexanol, which generate green and alcoholic notes (Curioni and Bosset, 2002), were probably arisen in the PC cheese from the metabolism of LAB strains. The amount of alcohol in cheese A samples could be related to the presence of *L. lactis* species. It is noteworthy that certain strains of lactococci can produce branched chain aldehydes and alcohols starting from valine, isoleucine and leucine (Christensen et al., 1999). These compounds have been reported as major alcohol of artisanal cheese such as Pecorino (Randazzo et al., 2008), La Serena (Carbonell et al., 2002), and Castelo Branco (Ferreira et al., 2009).

Only 2 aldehydes, nonanal and benzaldehyde were detected throughout cheese ripening, which were probably derived from microbial degradation of amino acids (transamination followed by decarboxylation) or from Strecker degradation, or from lipid oxidation. In general, they were not present in big quantities because they were rapidly converted into alcohols or into the corresponding acids. These aldehydes are generally characterized by green-like and herbaceous aromas (Moio et al., 1993).

Among linear fatty acid compounds, total it should be pinpointed that their content varied considerably between samples. In fact, butanoic, hexanoic, octanoic acids achieved the highest concentration in cheese A samples at the beginning of the ripening, while in cheese B the amounts increased significantly throughout the process. This variation may be due to differences in processing between the factories of origin and, perhaps, differences in the initial level of lypolysis in the milk used in cheese manufacture (Poveda and Cabezas, 2006). The frequency of hexanoic acid in cheeses probably indicates enhanced hexanoic-specific lipase activities from wild LAB strains coming from raw milk or lipase of artisanal rennets. Linear FFAs, containing four or more carbon atoms, are generally produced from lypolysis of milk fat and the lipases responsible for this process originate from the milk itself, moulds, LAB and/or propionibacteria (McSweeney and Sousa, 2000). Nevertheless, they can also be a result of the metabolism of lactose, biosynthesized directly from acetyl-CoA, or formed from amino acid conversion (Tavaria et al., 2004). These compounds are related to cheesy, sharp, and sweat-like odors and play an important role in the flavour formation of many cheese types as Camembert, Cheddar, Grana Padano, Pecorino, Ragusano and Roncal cheese (Curioni and Bosset, 2002). FFAs are important components of cheese aroma, either directly by their aromatic notes, or as precursors of carbonyl compounds, alcohols, alkanes, and esters.

Ketones are intermediate compounds which may be reduced to secondary alcohols. Methyl ketones are produced from fatty acids by oxidative degradation. The formation of methyl ketones is a result of enzymatic oxidation of fatty acids to β-ketoacids, which are then decarboxylated to corresponding methyl ketones with one carbon atom less (McSweeney and Sousa, 2000). Various aroma notes are associated with methyl ketones, such as 2-heptanone (musty, sweet, mouldy, varnish), detected only in the cheese B, and 2-nonanone (floral, fruity, peach), as a result of LAB metabolism. Methyl ketones are the principal compounds responsible for the flavour of different types of cheese: blue cheese (Engels et al., 1997), Gouda, Cheddar (Ziino et al., 2005) and Parmigiano cheese (Bellesia et al., 2003) and several raw milk cheeses (Carbonell et al., 2002; Ferreira et al., 2009).

It is interesting to highlight that terpens were detected only in cheese A samples. In particular, monoterpenes, such as limonene and α-pinene (Table 5), which are often associated to citrus and pine odors, respectively, are well-known components of plant essential oils, especially citrus species that are widely diffused in Sicily and Calabria (Dugo and Di Giacomo, 2002). These compounds are transferred to milk, but their influence in cheese flavour and aroma formation remains still controversial. Since the terpenes partly derived from livestock's fodder (Viallon et al., 1999), the large differences of terpenes concentration among cheeses could be explained by the different types of forage mixture.

Sulphur containing compounds primarily arise from the biodegradation of the sulphur/carbon bond of methionine or cysteine by the cheese microbial population. In this regard, bacteria such as lactobacilli and lactococci are believed to play an important role in sulphur compounds biosynthesis (Bonnarme et al., 2000). The methane-thiobis production, which was registered in both cheese samples, is probably due to the methanethiol oxidation (Landaud et al., 2008).

In conclusion, combining culture-dependent and -independent approaches, the present study provides a view of bacterial composition of Pecorino Crotonese cheese, despite the analyzed cheese samples were provided by only two farmers. Results highlighted the appearance of several LAB species during cheese ripening, which may contributed to the flavour formation of the final product.

Acknowledgements

The authors kindly thank Dr. Davide Rosa, Dr. Serena De Luca and Mr. Filippo Tomaselli for their precious contribution.

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Food Microbiology 32 (2012) 87–96

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Diversity of bacterial population of table olives assessed by PCR-DGGE analysis

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ARTICLE INFO

Article history:
Received 25 November 2011
Received in revised form
22 April 2012
Accepted 24 April 2012
Available online 8 May 2012

Keywords:
Lactic acid bacteria
Brine fermentation
Nocellara Etnea
Geracese cultivar
Culture-independent approach

ABSTRACT

Nocellara Etnea and Geracese table olives are produced according to traditional process, in which lactic acid bacteria (LAB) and yeasts are the dominant microorganisms. With the aim to evaluate the effect of selected starter cultures on dynamics of bacterial population during fermentation and on growth/survival of *Listeria* spp. artificially inoculated into the olive brine, a polyphasic approach based on the combination of culturing and PCR-DGGE analysis was applied.

Plating results showed a different concentration of the major bacterial groups considered among cultivars and the beneficial effect of LAB starters, which clearly inhibited *Enterobacteriaceae*. Moreover, results indicated that the brine conditions applied did not support the growth/survival of *Listeria monocytogenes* strain, artificially inoculated, highlighting the importance of selecting right fermentation parameters for assuring microbiological safety of the final products. Comparison of DGGE profile of Nocellara Etnea and Geracese table olives, displayed a great difference among cultivars, revealing a wide biodiversity within *Lactobacillus* population during Geracese olives fermentation. Based on cloning and sequencing of the most dominant amplicons, the presence, among others, of *Lactobacillus paracollinoides* and *Lactobacillus coryniformis* in Geracese table olives was revealed in table olives for the first time.

1. Introduction

Table olives are the most popular fermented vegetables in the Western world and a main part of the Mediterranean diet together with olive oil. Their production has been estimated approximately as 2 million tonnes, in the 2006–2007 crop year, with Spain, Italy and Greece countries being the main producers (IOOC, 2008). Table olives are produced from specifically cultivated fruit varieties harvested at the pre-determined stage of maturation. Two main methods are used to produce fermented table olives the Spanish method, for green olives (De Castro et al., 2002) and the Greek method, for black olives (Tassou et al., 2002). For producing naturally fermented table olives several intrinsic and extrinsic factors related to brine composition influence the fermentation process (Garrido Fernández et al., 1995), which is obtained without any prior debittering treatment. The composition of the microbial community and its dynamics throughout fermentation are crucial for determining the quality of the final product (Garrido Fernández et al., 1997; Chamkha et al., 2008). Lactic acid bacteria (LAB) are part of the indigenous microbial community of olives and species belonging to *Lactobacillus* genus are predominant during olives fermentation, whereas *Leuconostoc, Streptococcus, Enterococcus,* and *Pediococcus* are present in lower concentrations (Randazzo et al., 2010). It is commonly recognized that the natural process leads to unpredictable and longer fermentation as well as low quality products with variable sensory characteristics. Interest in the development and use of starter cultures for table olive fermentation is increasing because an appropriate inoculation of LAB can help to achieve a more controlled process, reducing debittering time and improving the sensorial and hygienic quality of the final product (Servili et al., 2006; Panagou et al., 2008). Several authors demonstrated that brine environment supports the growth/survival of several pathogen microorganisms such as *Listeria monocytogenes* (Caggia et al., 2004), and *Escherichia coli* O157:H7 (Spyropoulou et al., 2001). Moreover, spores of *Clostidium botulinum* were detected both in pasteurized and sterilized olives (Pereira et al., 2008), indicating that the technological parameters applied were not suitable to guarantee the microbiological safety of the product. More recently, the species *Enterobacter cloacae*, an opportunistic pathogen for humans, has been recovered in spontaneously fermented table olives (Bevilacqua et al., 2010).

On the basis of these results the main concern of industry could be to assure the safety aspect of final product throughout a full description of microbial ecosystem and its dynamics during olive fermentation process. Up to now, methods available for detection

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doi:10.1016/j.fm.2012.04.013

and identification of microbial population involved in table olive fermentation are very limited and generally culture-dependent, not providing reliable information on the composition of the entire microbial community. Denaturing Gradient Gel Electrophoresis (DGGE) of community amplicons obtained from 16S rRNA gene (PCR-DGGE) (Muyzer et al., 1993) has proven to be a versatile method to assess the biodiversity and population dynamics occurring in various fermented food such as cheese, wine, and meat (Randazzo et al., 2009a,b). In the present study, a PCR-DGGE approach was optimized to analyze microbial populations involved in table olive fermentation produced in different regions of Southern Italy with the aims to i) evaluate the effect of selected starter cultures on dynamics of bacterial population during fermentation and ii) investigate the growth/survival of *Listeria* spp. artificially inoculated into the olive brine.

2. Materials and methods

2.1. Olive samples

Table olives cv. Nocellara Etnea (N) were kindly provided from a table olive industry, located in Sicily. Olives cv. Geracese (G) were provided from a local table olive industry of Calabria, Italy. Samples from both olive types, from 2006 crops, were transported immediately after harvest to the laboratory of Microbiology of the DISPA. Olives were washed with tap water and subject to further treatments.

2.2. Origin of strains and preparation of cell cultures inoculum

Two selected strains of LAB, *Lactobacillus plantarum* UT 2.1 and *Lactobacillus casei* T19 belonging to the DISPA microbial collection, previously isolated from wine and olive brine, respectively (Randazzo et al., 2004, 2007) were used. Both strains were chosen for their acid production, for their ability to grow at different pH values and salt concentrations and for β-glucosidase activity, which is considered relevant for natural debittering of olives. Single frozen concentrated cultures of the two selected strains were grown at 32 °C in 50 ml of MRS broth supplemented with 4.5% (w v^{-1}) of NaCl to allow adaptation of strain cultures to the saline environment of the brine (De Castro et al., 2002). When the OD_{600} reached the value of 1.0, cells were harvested (8000 *g* for 10 min), washed and re-suspended in physiological water (0.9% w v^{-1} NaCl) and added to brines to have a final concentration of 8 $\log_{10}$ cfu ml^{-1}. Un-inoculated brine samples were used as control.

L. monocytogenes OML45 strain, previously isolated from brine olives (Caggia et al., 2004), belonging to the DISPA microbial collection, was also used for inoculation. The strain was maintained on Tryptic Soy Agar (TSA) (CM 129, Oxoid, Basingstoke, UK) slants at 5 °C. Cell culture of *L. monocytogenes* OLM45, grown in Typtic Soy Agar medium (Oxoid) containing 0.6% of Yeast Extract at 32 °C until the log-phase stage, was centrifuged at 5590 *g* for 10 min and then the pellet was re-suspended in a physiological solution (0.9% NaCl) and maintained until use. The initial bacterial inoculum (6 $\log_{10}$ cfu ml^{-1}) was added to vessels. The cell concentration of the inoculum was determined by plating on TSA followed by incubation at 32 °C for 24 h.

2.3. Table olive processing

Nine kilograms of fruits were processed using an experimental semi-industrial manufacture, using a 20 l total capacity screw-capped PVC vessels. Briefly, after preliminary treatments (selection and calibration) olives were immersed in 10 l of fresh brine solution, containing 5% (w v^{-1}) of NaCl, previously sterilized in order to reduce brine contaminations and to standardize starter inocula. One week later the brines were inoculated with the mixed LAB starter culture previously described, and few days after with *L. monocytogenes* strain. In total 4 vessel samples (from I to IV) were produced in duplicate for each olive cultivar, as reported in Table 1. All fermentation vessels were kept at room temperature (about 20 °C) for an overall period of 180 days. Vessels were initially kept semi-closed to allow the initial growth of yeasts and only later totally closed. Brine pH values were continuously monitored using a pH meter (H19017, Microprocessor, Hanna Instruments) and adjusted, for the first 7 days, by adding food grade lactic acid up to a final value below 5. During the first 60 days of fermentation coarse salt, were weekly added up to 6% in order both to keep constant the salt concentration and to allow a steady adaptation to the brine environment of inoculated LAB. Fresh brine was periodically supplied in order to maintain olives totally dipped to avoid the moulds growth.

2.4. Microbiological analyses

Microbiological analyses of brines were performed, in duplicate, at 0, 7, 15, 30, 60, 90, 120, and 180 days of fermentation. Brine samples (1 ml) were aseptically transferred to 9 ml of sterile quarter-strength Ringer's solution (QRS). Decimal dilutions in QRS were prepared and plated into following agar media (all from Oxoid, Milan, Italy): Plate Count Agar for mesophilic bacteria counts, incubated at 32 °C for 24–48 h; de Man-Rogosa-Sharp adjusted to pH at 5.4 containing cycloheximide (Sigma) (100 mg l^{-1}), for LAB, incubated under anaerobic conditions at 32 °C for 48–72 h; Violet Red Bile Glucose Agar for the enterobacteria counts, incubated anaerobically at 37 °C for 24–48 h; MSA media for the enumeration of staphylococci, incubated at 32 °C for 48 h; Sabouraud Dextrose Agar (Oxoid, CM41) for yeasts and moulds, incubated at 25 °C for 4 days. Growth data from plate counts were enumerated as $\log_{10}$ values. *Listeria* spp. was enumerated following the official MPN methods, using the *Listeria* enrichment broth base (Oxoid) previously sterilized and added with supplement as described by Caggia et al. (2004).

2.5. Physico-chemical analyses

The pH values of brines were continuously measured using a pH meter, the NaCl concentration was monitored by titrating brine samples (5 ml) using a standardized solution of silver nitrate (0.1 N) and potassium chromate (5%, w v^{-1}) as indicator (Garrido Fernández et al., 1997). Titratable acidity was determined by titration with sodium hydroxide (0.1 N) and expressed as mEq l^{-1} brine; chlorides were determined by $AgNO_3$ titration according to the Mohr method and expressed as g 100 g^{-1}.

Total polyphenols were extracted from olives following the method reported by Amiot et al. (1986), measured spectrophotometrically at 725 nm after reaction with the Folin–Ciocalteu's

Table 1
Olives cultivar samples and type of inoculum used in the present study.

Olives cultivar	Vessel samples	Type of inoculum
Nocellara Etnea	N_I	Un-inoculated olives: control
	N_{II}	*L. plantarum* plus *L. casei*
	N_{III}	*L. plantarum* plus *L. casei* plus *L. monocytogenes*
	N_{IV}	*L. monocytogenes*
Geracese	G_I	Un-inoculated olives: control
	G_{II}	*L. plantarum* plus *L. casei*
	G_{III}	*L. plantarum* plus *L. casei* and *L. monocytogenes*
	G_{IV}	*L. monocytogenes*

reagent, and expressed as mg kg^{-1} of gallic acid by mean of a calibration plot using pure gallic acid (Sigma–Aldrich, Germany) as standard.

2.6. HLPC analysis of brine samples

HPLC analysis of phenol fraction of brine samples was achieved by directly injecting the filtered brine in the chromatographic system. HPLC analysis was conducted using a Knauer HPLC system (Smartline Pump 1000) equipped with Waters 486 UV detector set at 280 nm. A C18 monomeric 120 Å, 5 μm particle size, 4.6 × 250 mm column (Grace Vydac, Denali) fitted with 4.6 mm guard column were used. The solvent flow rate was 1.0 ml min^{-1}. Separation was achieved by elution gradient using an initial composition of 90% of A solution, water acidified with 2% acetic acid (Riedel-de Haën, Germany) and 10% of B solution, methanol (Sigma–Aldrich, Germany). After 15 min of isocratic conditions, the concentration of B solution was increased to 30%, with further stepwise increases to 40% B at 25 min, 70% B at 35 up to 40 min, hold for 5 min and return to initial conditions over 5 min. The phenolic compounds were identified by comparing retention times with pure oleuropein (Extrasynthese) or hydroxytyrosol obtained by acid hydrolysis of oleuropein. The response factor of hydroxytyrosol was considered the same of tyrosol.

2.7. Statistical analysis

All experiments were performed on duplicate and the experimental data were reported as average values and provided with Standard Deviation or Standard Error (Figs. 1 and 2). All analyses were performed using General Linear Models (GLM) repeated measures with SPSS for Windows (version 12.0) in order to assess the time effect on the different treatments of the microbial groups, evaluated using different selective medium, across the fermentation period (0–180 days), within the same cultivar and among the two cultivars considered. Differences from mean values were evaluated using Duncan's test. Significance was tested with Wilks Lambda. Differences among time sampling, for each selective medium used, were statistically assessed throughout multiple range test.

2.8. Total genomic DNA extraction from reference strains, from bacterial cultures and from brine samples

L. plantarum DSMZ 20246, *Lactobacillus brevis* DSMZ 20054, *Lactobacillus fermentum* DSMZ 20052, *Lactobacillus paracasei* LMG 23516, *Streptococcus thermophilus LMG 11164*, purchased from international microbial collections, were used as reference strains. Genomic DNA from both reference strains and bacterial isolates, was extracted from 6 ml of overnight cultures grown in MRS broth as described by Gala et al. (2008). For the extraction and purification of total DNA directly from brine samples, the QIAamp DNA mini kit (QIAGEN, Milan, Italy) was used, following the instruction procedures.

2.9. PCR amplification for DGGE analysis

PCR amplification was performed in a 50 μl volume using a GenAmp PCR System 9700 (Perkin–Elmer, Foster City, CA, USA). The reaction mixtures consisted of 1.25 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 50 μM dNTPs, 5 pmol primers each and 1 μl of properly diluted template DNA. The reaction mixture with no template DNA was used as a negative control. To investigate the dominant bacterial communities by DGGE analysis PCR products were generated using universal primers U968-GC and L1401-r to amplify the V6 to V8 region of eubacterial 16S rDNA (Nubel et al., 1996). The 40-nucleotide GC rich sequence at the 50 end of primer U968-GC improves the detection of sequence variations of amplified DNA fragments by subsequent TGGE/DGGE running (Muyzer et al., 1993). The samples were amplified in a Perkin Elmer Applied Biosystem GenAmp PCR System 9700 (Foster City, CA, USA) programmed as follows: initial denaturation of DNA for 5 min at 94 °C; 35 cycles each consisting of 30 s at 94 °C, 30 s at 56 °C and 40 s at 68 °C; and extension of incomplete products for 7 min at 68 °C.

To confirm an insert of the correct size, PCR using the cell lysates as template was performed with primer pairs 7-f and 1510-r (Lane, 1991) and T7 and Sp6 (Promega Corporation, Madison, USA) to amplify the bacterial 16S rRNA gene prior to cloning and sequence analysis. DNA amplification was carried out with the reaction mixtures as described above under the following conditions: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 1.5 min; and finally 68 °C for 7 min.

PCR products were quantified by electrophoresis on a 1.2% (w v^{-1}) agarose gel containing ethidium bromide, and where necessary, were purified with the Qiaquick PCR purification kit according to the manufacturer's instructions.

2.10. DGGE analysis

DGGE analysis of PCR amplicons was performed on the Dcode System apparatus (BioRad, Hercules, CA), as previously described (Muyzer et al., 1993). Samples were loaded into an 8% (w v^{-1}) polyacrylamide gel (acrylamide:bisacrylamide 37.5 : 1) in 0.5× TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA pH 8.0).

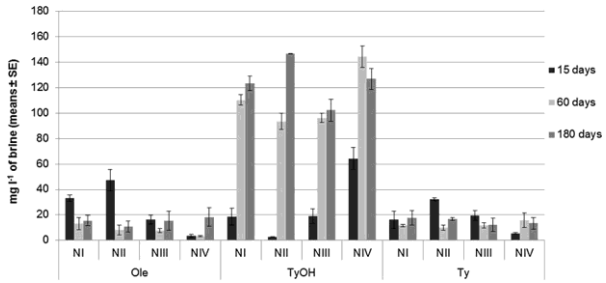

Fig. 1. Phenol content, expressed as mg l^{-1} of Nocellara Etnea brine samples, evaluated throughout fermentation.

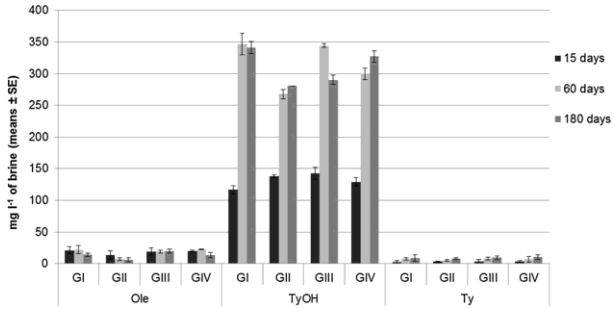

Fig. 2. Phenol content, expressed as mg l^{-1} of Geracese brine samples, evaluated throughout fermentation.

Optimal separation was achieved with 30–60% urea–formamide denaturant gradient, increasing in the direction of electrophoresis. A 100% denaturant corresponds to 7 M urea and 40% (v v^{-1}) formamide. Electrophoresis was performed at a constant voltage of 75 V and at temperature of 60 °C for 16 h. The DNA bands were visualized by silver staining and developed as previously described (Sanguinetti et al., 1994).

2.11. Cloning in plasmid inserts and sequencing of 16S rRNA gene

Clone libraries of the 16S rRNA gene amplicons from brines of Nocellara Etnea and Geracese cultivars taken at 30 and 120 days of fermentation (samples NII, GII, NVI and GIV) respectively were constructed. Amplicons derived from PCR of the 16S rRNA gene using primer pairs 7-f and 1510-r were purified and cloned in *E. coli* JM109 using the pGEM-^{T} plasmid vector system (Promega, Madison, USA) in accordance with the manufacturer's instructions. The transformants were amplified and their mobility was compared to the rDNA-derived patterns of brine samples by DGGE (data not shown). The clones that produced a single DGGE amplicon with a melting position identical to that one of the dominant bands in the brine samples DNA patterns were sequenced by Biodiversity s.p.a. (Brescia, Italy) company. To determine the closest known relatives of the isolates, partial 16S rRNA gene sequences were compared to those in the GenBank database (http://ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) using BLAST program. Sequences with a percentage identity of 99% or greater were considered to belong to the same species.

3. Results

3.1. Microbiological analysis

The mean of microbial counts and standard deviation obtained by classical enumeration of bacterial population present in the Nocellara Etnea (N) and Geracese (G) cultivar olive samples differently treated, during brine fermentation are shown in Tables 2 and 3, respectively.

Overall, analyzing Nocellara Etnea olives, the initial LAB concentration was quite different among the brine samples analyzed, and as expected, the highest counts were registered in the samples inoculated with LAB starters. Similarly, LAB dynamic throughout brine fermentation was different among samples. In detail, in the un-inoculated sample (control, sample N_I), LAB counts significantly increased up to 15 days of fermentation, maintaining a constant value (approximately 5 $\log_{10}$ cfu ml^{-1}) up to 120 days. Similar trend was observed in samples inoculated with LAB starter and *L. monocytogenes* (sample N_{III}) and in samples inoculated only with *L. monocytogenes* species (sample N_{IV}). In samples inoculated with starters (sample N_{II}), LAB count was quite constant up to 15 days, reaching an abnormal value of 11 $\log_{10}$ cfu ml^{-1}, at the 30th day of fermentation and showing a significant decrease until the end of the process. In general in the latest sample the final LAB concentration was 2 $\log_{10}$ units higher than that registered in the other samples.

Mesophilic aerobic bacteria counts showed a similar trend in all samples increasing at the 30th day of 2 units respect to the initial values.

Staphylococci counts exhibited similar trend among brine samples, with a significant increase (close to 5 l $\log_{10}$ cfu ml^{-1}) up to 30 days of fermentation.

The initial level of yeasts was very low (less than 2 $\log_{10}$ cfu ml^{-1}) and their dynamics throughout fermentation was quite similar among samples, showing an increasing trend (up to 5 $\log_{10}$ cfu ml^{-1}) after 30 days of fermentation and a slightly decrease until the end of fermentation (Table 2).

No *Enterobacteriaceae* was registered at the end of the process in any samples. Nevertheless, while in treated samples the level significantly decreased after 60 days, in the control (sample N_I) the count reached almost 7 $\log_{10}$ cfu ml^{-1} at 10th day of fermentation (Table 2).

It is interesting to note that no *Listeria* spp. colony was detected during the whole fermentation process in any studied samples, even in those where it was artificially inoculated at the beginning of fermentation.

Regarding microbial populations of brine samples of Geracese olives results are shown in Table 3. In general we can assert that microbial population resulted higher than those registered for Nocellara Etnea olives (Table 3). Higher level of all detected microbial groups were found at the initial time revealing this cultivar as a richer source of epiphytic microbial population. In detail, LAB counts initially were approx 6 log cfu ml^{-1} in the control (G_I) and in samples inoculated with *L. monocytogenes* (G_{IV}) and, as expected, were close to 8 $\log_{10}$ cfu ml^{-1} in the samples inoculated with LAB cultures (G_{II} and G_{III}) (Table 3). Moreover, while the LAB community was quite constant throughout fermentation in the sample G_I and G_{IV}, in the samples G_{II} and G_{III} it exhibited a significant increase reaching values of about 9 $\log_{10}$ cfu ml^{-1}. Mesophilic aerobic bacteria showed similar trend in all samples analyzed, reaching the highest value after 30 days of fermentation except in sample inoculated with LAB starters (sample G_{II}) where the highest values (about 7 $\log_{10}$ cfu ml^{-1}) were detected at the 15th day

Table 2
Mean $\log_{10}$ of bacterial population counts during fermentation of Nocellara Etnea olives.

Olive samples	Fermentation time (days)	Microbial log counts (expressed as mean of $\log_{10}$ cfu ml^{-1}) and standard deviations (SD)				
		MRS Lactic acid bacteria	PCA Mesophilic bacteria	MSA Staphylococci	SAB Yeasts	VRBGA *Enterobacteiaceae*
N_I	0	$2.80^{a} \pm 0.57$	$3.41^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$1.16^{a} \pm 1.64$	$3.59^{c} \pm 0.08$
	7	$3.39^{ab} \pm 0.55$	$3.72^{b} \pm 0.00$	$0.00^{a} \pm 0.00$	$3.51^{b} \pm 1.46$	$5.30^{d} \pm 0.32$
	15	$5.75^{c} \pm 0.28$	$5.48^{c} \pm 0.00$	$4.00^{b} \pm 0.00$	$4.21^{bc} \pm 0.37$	$6.78^{e} \pm 0.03$
	30	$5.50^{c} \pm 0.70$	$5.90^{d} \pm 0.00$	$5.20^{c} \pm 0.00$	$6.01^{c} \pm 0.65$	$5.58^{d} \pm 0.09$
	60	$5.46^{c} \pm 0.48$	$5.48^{c} \pm 0.09$	$5.15^{c} \pm 0.00$	$5.34^{bc} \pm 0.49$	$2.06^{b} \pm 0.51$
	120	$5.90^{c} \pm 0.42$	$5.42^{c} \pm 0.10$	$4.01^{b} \pm 0.23$	$5.46^{bc} \pm 0.05$	$0.49^{a} \pm 0.69$
	180	$4.62^{bc} \pm 0.54$	$6.08^{d} \pm 0.25$	$0.00^{a} \pm 0.00$	$4.86^{bc} \pm 0.30$	$0.00^{a} \pm 0.00$
	Sig.§	**	**	**	**	**
N_{II}	0	$7.62^{bc} \pm 0.34$	$5.24^{a} \pm 0.13$	$0.00^{a} \pm 0.00$	$1.88^{a} \pm 0.16$	$3.36^{c} \pm 0.20$
	7	$6.69^{abc} \pm 0.16$	$6.43^{b} \pm 0.16$	$0.00^{a} \pm 0.00$	$2.34^{a} \pm 0.48$	$3.19^{c} \pm 0.19$
	15	$7.74^{c} \pm 0.94$	$6.49^{b} \pm 0.01$	$4.00^{b} \pm 0.00$	$3.63^{b} \pm 0.17$	$2.25^{b} \pm 0.09$
	30	$11.32^{d} \pm 1.44$	$7.95^{c} \pm 0.73$	$5.55^{d} \pm 0.42$	$5.56^{c} \pm 0.30$	$0.56^{a} \pm 0.79$
	60	$6.00^{ab} \pm 0.00$	$8.99^{d} \pm 0.12$	$4.89^{cd} \pm 0.20$	$5.16^{c} \pm 0.02$	$0.00^{a} \pm 0.00$
	120	$5.32^{a} \pm 0.00$	$5.22^{a} \pm 0.21$	$4.27^{bc} \pm 0.03$	$4.86^{c} \pm 0.79$	$0.00^{a} \pm 0.00$
	180	$6.82^{abc} \pm 0.02$	$6.13^{b} \pm 0.41$	$4.90^{cd} \pm 0.61$	$4.65^{c} \pm 0.00$	$0.00^{a} \pm 0.00$
	Sig.	**	**	**	**	**
N_{III}	0	$5.56^{b} \pm 0.12$	$4.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$1.75^{a} \pm 0.12$	$4.49^{d} \pm 0.53$
	7	$7.20^{cb} \pm 0.08$	$5.45^{bc} \pm 0.21$	$0.00^{a} \pm 0.00$	$5.29^{c} \pm 0.01$	$3.44^{c} \pm 0.16$
	15	$7.31^{de} \pm 0.01$	$5.50^{bc} \pm 0.70$	$0.00^{a} \pm 0.00$	$5.64^{d} \pm 0.07$	$4.07^{cd} \pm 0.09$
	30	$7.08^{c} \pm 0.12$	$6.15^{c} \pm 0.03$	$3.28^{c} \pm 0.02$	$5.00^{bc} \pm 0.00$	$2.46^{b} \pm 0.32$
	60	$7.43^{e} \pm 0.55$	$6.99^{d} \pm 0.05$	$2.59^{b} \pm 0.14$	$5.06^{bc} \pm 0.32$	$0.27^{a} \pm 0.28$
	120	$4.97^{a} \pm 0.33$	$5.32^{b} \pm 0.39$	$3.84^{d} \pm 0.03$	$4.89^{b} \pm 0.07$	$0.00^{a} \pm 0.00$
	180	$4.80^{a} \pm 0.32$	$5.83^{bc} \pm 0.08$	$4.65^{c} \pm 0.07$	$5.27^{c} \pm 0.03$	$0.00^{a} \pm 0.00$
	Sig.	**	**	**	**	**
N_{IV}	0	$2.21^{a} \pm 0.12$	$1.73^{a} \pm 0.36$	$0.00^{a} \pm 0.00$	$1.47^{a} \pm 0.73$	$3.54^{d} \pm 0.60$
	7	$4.16^{b} \pm 0.65$	$3.34^{b} \pm 0.55$	$0.00^{a} \pm 000$	$1.77^{a} \pm 0.73$	$3.05^{cd} \pm 0.09$
	15	$4.13^{b} \pm 0.62$	$3.38^{b} \pm 0.12$	$4.76^{b} \pm 0.11$	$3.93^{b} \pm 0.08$	$2.55^{c} \pm 0.16$
	30	$4.30^{b} \pm 0.42$	$4.97^{c} \pm 0.28$	$5.70^{c} \pm 0.07$	$4.45^{bc} \pm 0.03$	$1.50^{d} \pm 0.39$
	60	$4.40^{b} \pm 0.57$	$4.86^{c} \pm 0.18$	$5.34^{bc} \pm 0.49$	$4.58^{bc} \pm 0.15$	$0.39^{a} \pm 0.55$
	120	$4.74^{b} \pm 0.04$	$5.36^{c} \pm 0.26$	$5.46^{c} \pm 0.36$	$5.46^{c} \pm 0.09$	$0.32^{a} \pm 0.45$
	180	$4.67^{b} \pm 0.78$	$5.10^{c} \pm 0.21$	$5.44^{c} \pm 0.37$	$5.13^{c} \pm 0.20$	$0.00^{a} \pm 0.00$
	Sig.	*	**	**	**	**

abc means, for each medium, in the same column followed by different lowercase letters are significantly different.
Sig.§: Significance during fermentation within each sample; * for $P \leq 0.05$; ** for $P \leq 0.01$.

(Table 3). Staphylococci exhibited a similar trend among samples, with a significant decrease throughout the fermentation (Table 3). The initial level of yeasts was approximately 3 $\log_{10}$ cfu ml^{-1} in all samples. Throughout fermentation process the level significantly increased, reaching the value of 5–7 $\log_{10}$ cfu ml^{-1} in all brine samples and decreased only at the end of fermentation. The level of *Enterobacteriaceae* was about 3–4 $\log_{10}$ cfu ml^{-1} at the beginning of the fermentation, then showed a rapid decrease in samples inoculated with LAB starters (G_{II} and G_{III}), while in the other samples increased up to 30 days and significantly decreased until the end of fermentation.

Also in this case no colonies belonging to *Listeria* spp. were detected in any brine samples analyzed even in those artificially inoculated with the pathogen (data not shown).

3.2. Physico-chemical results

Changes in physico-chemical parameters during brine fermentation of Nocellara Etnea and Geracese cultivar olives are reported in Tables 4 and 5, respectively. Evaluating physico-chemical change of Nocellara Etnea olive brines, results showed a significant increase of titratable acidity in all samples analyzed throughout the fermentation, registering a final value between 70 and 85 mEq l^{-1}. Moreover the rate of acidification was higher in the samples inoculated with LAB starter and LAB plus *L. monocytogenes* than in the control one. Moreover, GLM data, assessed to evaluate the effect of the fermentation on titratable acidity, revealed significant differences among olive samples differently treated (for Wilk's lambda $F = 4127.66$; $P < 0.0001$) while the trend between cultivars was similar throughout the fermentation (for Wilk's lambda $F = 4127.66$; $P < 0.0001$) (data not shown). Regarding pH values, results revealed a significant decrease in all samples, especially in that inoculated with LAB starter (N_{II}), which exhibited the lowest final value (pH 3.10) (Table 4). The initial level of salt concentration was around 5% and showed a significant decrease followed by an increase during fermentation due to salt addition, registering a final value of about 6.6–6.8% in both cultivars without significant differences (data not shown).

Physico-chemical results of Geracese cultivar olives exhibited similar trend to Nocellara Etnea olives, except for the pH values which registered a lower decrease during the fermentation (Table 5). It is interesting to note that sample inoculated with LAB (sample G_{II}) showed the lowest value, with any significant decrease during the whole fermentation period. Also the samples G_{III} exhibited any significant decrease during fermentation with a final value of pH 4.0 (Table 5).

Results of phenol content of Nocellara Etnea and Geracese olive brines are shown in Figs. 1 and 2, respectively. The dynamics of oleuropein content as well as the other compounds were quite different among samples. Following Nocellara cv olives, the oleuropein content showed a significant decrease during fermentation in the control and in the samples inoculated with LAB starters (N_I and N_{II}, respectively). Diversely in the samples inoculated both with LAB and *L. monocytogenes* (N_{III}) and inoculated only with *L. monocytogenes* (N_{IV}), oleuropein content exhibited a slight decrease up to 60 days and an increase until the end of the

Table 3
Mean $\log_{10}$ of bacterial population counts during fermentation of Geracese olives.

Olives samples	Fermentation time (days)	Microbial log counts (expressed as mean of $\log_{10}$ g cfu ml^{-1}) and standard deviations (SD)				
		MRS Lactic acid bacteria	PCA Mesophilic bacteria	MSA Staphylococci	SAB Yeasts	VRBGA *Enterobacteiaceae*
G_I	0	$6.07^{a} \pm 0.75$	$5.00^{a} \pm 0.00$	$3.17^{c} \pm 0.00$	$3.00^{a} \pm 0.01$	$3.53^{c} \pm 0.01$
	7	$6.59^{a} \pm 0.03$	$6.42^{b} \pm 0.03$	$5.66^{d} \pm 0.22$	$5.17^{c} \pm 0.01$	$5.16^{d} \pm 0.12$
	15	$6.69^{a} \pm 0.00$	$7.03^{bc} \pm 0.03$	$3.00^{c} \pm 0.01$	$5.45^{cd} \pm 0.04$	$6.80^{e} \pm 0.05$
	30	$6.47^{a} \pm 0.67$	$7.20^{bc} \pm 0.29$	$3.00^{c} \pm 0.01$	$6.03^{e} \pm 0.05$	$5.65^{d} \pm 0.18$
	60	$6.23^{a} \pm 0.33$	$6.96^{bc} \pm 0.83$	$1.00^{b} \pm 0.01$	$5.77^{de} \pm 0.10$	$2.08^{b} \pm 0.55$
	120	$6.30^{a} \pm 0.01$	$7.60^{cd} \pm 0.00$	$0.00^{a} \pm 0.00$	$5.69^{de} \pm 0.00$	$0.00^{a} \pm 0.00$
	180	$5.65^{a} \pm 0.66$	$8.04^{d} \pm 0.08$	$0.00^{a} \pm 0.00$	$4.46^{b} \pm 0.48$	$0.00^{a} \pm 0.00$
	Sig.	ns	**	**	**	**
G_{II}	0	$8.50^{ab} \pm 0.70$	$5.00^{a} \pm 0.00$	$3.35^{e} \pm 0.25$	$2.88^{a} \pm 0.15$	$3.35^{c} \pm 0.25$
	7	$8.60^{abc} \pm 0.00$	$6.37^{ab} \pm 1.60$	$3.00^{d} \pm 0.00$	$5.00^{b} \pm 0.00$	$3.15^{c} \pm 0.21$
	15	$9.56^{d} \pm 0.05$	$7.43^{b} \pm 0.08$	$4.72^{f} \pm 0.17$	$5.08^{b} \pm 0.01$	$2.15^{b} \pm 0.21$
	30	$9.17^{bcd} \pm 0.04$	$6.62^{ab} \pm 0.63$	$2.00^{c} \pm 0.00$	$5.56^{bc} \pm 0.37$	$0.00^{a} \pm 0.00$
	60	$9.49^{cd} \pm 0.64$	$6.45^{ab} \pm 0.03$	$0.00^{a} \pm 0.00$	$5.45^{bc} \pm 0.63$	$0.00^{a} \pm 0.00$
	120	$8.00^{a} \pm 0.00$	$6.30^{ab} \pm 0.00$	$1.00^{b} \pm 0.00$	$5.84^{c} \pm 0.00$	$0.00^{a} \pm 0.00$
	180	$8.34^{ab} \pm 0.31$	$6.61^{ab} \pm 0.23$	$1.00^{b} \pm 0.00$	$5.69^{bc} \pm 0.02$	$0.00^{a} \pm 0.00$
	Sig.	*	ns	**	**	**
G_{III}	0	$8.34^{a} \pm 0.75$	$5.24^{a} \pm 0.33$	$3.47^{b} \pm 0.00$	$3.40^{a} \pm 0.17$	$4.32^{b} \pm 0.29$
	7	$8.60^{a} \pm 0.01$	$6.42^{c} \pm 0.11$	$3.00^{b} \pm 0.00$	$5.00^{b} \pm 0.01$	$3.15^{ab} \pm 0.21$
	15	$9.56^{b} \pm 0.02$	$5.76^{abc} \pm 0.12$	$3.30^{b} \pm 0.42$	$5.61^{b} \pm 0.38$	$4.20^{b} \pm 1.02$
	30	$9.53^{b} \pm 0.33$	$8.46^{d} \pm 0.33$	$2.57^{b} \pm 0.81$	$7.20^{c} \pm 0.56$	$1.84^{ab} \pm 2.61$
	60	$9.57^{b} \pm 0.38$	$6.18^{bc} \pm 0.05$	$0.65^{a} \pm 0.91$	$5.34^{b} \pm 1.90$	$1.83^{ab} \pm 2.58$
	120	$9.77^{b} \pm 0.01$	$6.00^{bc} \pm 0.01$	$1.00^{a} \pm 0.00$	$4.00^{ab} \pm 0.01$	$0.00^{a} \pm 0.00$
	180	$9.15^{ab} \pm 0.26$	$5.51^{ab} \pm 0.55$	$1.10^{a} \pm 0.14$	$4.20^{ab} \pm 0.14$	$0.00^{a} \pm 0.00$
	Sig.	*	**	**	*	ns
G_{IV}	0	$6.60^{b} \pm 0.00$	$5.56^{a} \pm 0.78$	$3.47^{d} \pm 0.00$	$3.00^{a} \pm 0.00$	$3.68^{b} \pm 0.22$
	7	$6.60^{b} \pm 0.00$	$6.41^{a} \pm 0.00$	$3.00^{cd} \pm 0.00$	$4.66^{b} \pm 0.00$	$4.25^{b} \pm 1.34$
	15	$6.69^{b} \pm 0.00$	$6.69^{a} \pm 1.19$	$3.30^{d} \pm 0.42$	$5.47^{d} \pm 0.00$	$4.88^{b} \pm 2.67$
	30	$4.95^{a} \pm 0.49$	$8.21^{b} \pm 0.18$	$2.92^{cd} \pm 0.31$	$6.15^{f} \pm 0.21$	$0.50^{a} \pm 0.70$
	60	$6.61^{b} \pm 0.11$	$6.00^{a} \pm 0.31$	$1.99^{bc} \pm 0.97$	$5.80^{e} \pm 0.62$	$0.34^{a} \pm 0.49$
	120	$6.47^{b} \pm 0.00$	$6.00^{a} \pm 0.00$	$1.00^{ab} \pm 0.00$	$5.83^{e} \pm 0.00$	$0.50^{a} \pm 0.70$
	180	$6.54^{b} \pm 0.46$	$5.72^{a} \pm 0.56$	$0.84^{a} \pm 0.36$	$5.11^{c} \pm 0.14$	$0.50^{a} \pm 0.70$
	Sig.	**	*	**	**	*

abc: See Table 2; ns: not significant.

fermentation (Fig. 1). Concerning the hydroxytyrosol content all samples registered a significant increase throughout the fermentation reaching a final concentration between 100 and 150 mg l^{-1}. The tyrosol content was quite constant in the control sample, it decreased in the N_{II} and N_{III} samples and increased in the N_{IV} sample (Fig. 1). Statistical results showed significant differences for hydroxytyrosol ($P \leq 0.01$) and for oleuropein ($P \leq 0.05$) only respect to the time, while no differences respect to the treatments were revealed (data not shown).

Among Geracese olives (Fig. 2) the sample inoculated with LAB showed the lowest oleuropein content after 180 days, and significant difference among treatments ($P \leq 0.01$) (data not shown). Hydroxytyrosol content exhibited a significant increase during fermentation in all studied samples (Fig. 2) while tyrosol content was quite similar among samples. Comparing phenol content of the different cultivar olive samples, a notably difference in hydroxytyrosol value appears between the cultivars. The Geracese samples reached a content higher than 300 mg l^{-1}, while Nocellara Etnea registered a mean value of 146 mg l^{-1}. Moreover, the Geracese olives showed a polyphenol content (6055 mg kg^{-1}) higher than Nocellara Etnea cultivar (5290 mg kg^{-1}) as well as a lower sugar content (data not shown).

3.3. DGGE analysis of bacterial population throughout table olive fermentation

To investigate the diversity and dynamics of the dominant bacterial population of table olives, brine samples were taken at different days of fermentation (from 0 to 180 days) and were investigated by PCR-DGGE, using universal primers, which amplified the V6 to V8 region of the 16S rRNA gene. Results of PCR-DGGE profiles of Nocellara Etnea and Geracese cultivar olive samples taken at 0, 30 and 120 days are showed in Figs. 3 and 4, respectively. In general, bacterial community dynamics of Nocellara Etnea samples differently treated evaluated throughout fermentation process reflected in a stable DGGE profile, suggesting similarity in the bacterial composition. In detail, the un-inoculated olives samples (control, sample N_I) showed only one strong dominant band (band 1, lanes 1, 2 and 3, Fig. 3), and a weak band (band 2). An additional band (band 3) was visualized in the sample inoculated with starter cultures and in the samples inoculated with *L. monocytogenes* (sample N_{II} and N_{IV}) (lines 4, 5, 6, 10, 11 and 12, Fig. 3), which remained quite dominant throughout the fermentation process. Moreover, the band 3 was also detected in the sample inoculated with LAB and *L. monocytogenes* at 120th day of fermentation (line 9, Fig. 3). Other weak bands (bands 4 and 5) were also detected both in sample N_{II} and N_{IV}. Different profile was exhibited by sample inoculated both with starters and *L. monocytogenes* (sample N_{III}), which showed an increase in biodiversity during fermentation, revealed by the appearing of the weak bands 6 and 7, and of the dominant bands 8 and 9 (line 9, Fig. 3). A quite stable profile was revealed by the sample inoculated only with *L. monocytogenes* (sample N_{IV}, lines 10, 11, and 12, Fig. 3). Most of the amplicons dominated throughout the fermentation (bands 1, 2, 3, 4, 5, and 6, Fig. 3) while new bands (7, 8, and 9, Fig. 1), appeared only at 120th day of fermentation (line 12, Fig. 3).

In order to identify the most dominant bands in the DGGE profiles, reference bacterial strains were chosen as ladder and used

Table 4
Chemical change in brine during fermentation of Nocellara Etnea olives.

Olives samples	Fermentation time (days)	Titratable acidity (mEq l^{-1} brine)	pH	Salt concentration (g100 g^{-1})
N_I	0	$1.68^{a} \pm 0.72$	$5.32^{c} \pm 0.03$	$5.00^{b} \pm 0.00$
	7	$2.18^{ab} \pm 0.44$	$5.00^{c} \pm 0.06$	$4.46^{a} \pm 0.29$
	15	$4.87^{bc} \pm 0.14$	$4.59^{b} \pm 0.02$	$4.51^{a} \pm 0.22$
	30	$7.50^{c} \pm 0.00$	$4.47^{b} \pm 0.17$	$5.50^{c} \pm 0.14$
	60	$13.80^{d} \pm 1.69$	$3.95^{a} \pm 0.07$	$6.09^{d} \pm 0.02$
	120	$43.75^{e} \pm 1.76$	$3.89^{a} \pm 0.00$	$6.68^{e} \pm 0.04$
	180	$71.25^{f} \pm 1.76$	$3.78^{a} \pm 0.15$	$6.76^{e} \pm 0.05$
	Sig.	**	**	**
N_{II}	0	$1.97^{a} \pm 0.18$	$4.12^{c} \pm 0.14$	$5.00^{b} \pm 0.00$
	7	$5.00^{b} \pm 0.28$	$4.35^{c} \pm 0.07$	$4.51^{a} \pm 0.22$
	15	$8.84^{c} \pm 0.12$	$3.55^{b} \pm 0.07$	$4.65^{a} \pm 0.01$
	30	$12.50^{d} \pm 0.00$	$3.30^{a} \pm 0.14$	$5.58^{c} \pm 0.02$
	60	$17.25^{e} \pm 0.35$	$3.10^{a} \pm 0.14$	$6.14^{d} \pm 0.09$
	120	$34.45^{f} \pm 0.77$	$3.15^{a} \pm 0.07$	$6.72^{e} \pm 0.02$
	180	$75.00^{g} \pm 0.00$	$3.10^{a} \pm 0.02$	$6.70^{e} \pm 0.02$
	Sig.	**	**	**
N_{III}	0	$1.87^{a} \pm 0.14$	5.16 ± 0.05	$5.11^{b} \pm 0.14$
	7	$2.50^{a} \pm 0.00$	4.95 ± 0.07	$4.39^{a} \pm 0.38$
	15	$9.05^{b} \pm 0.21$	4.55 ± 0.07	$4.41^{a} \pm 0.36$
	30	$16.50^{c} \pm 1.41$	4.15 ± 0.04	$5.42^{b} \pm 0.25$
	60	$22.50^{d} \pm 0.00$	4.58 ± 0.36	$6.10^{c} \pm 0.04$
	120	$50.00^{e} \pm 0.00$	4.45 ± 0.62	$6.67^{d} \pm 0.04$
	180	$85.00^{f} \pm 0.70$	4.60 ± 0.21	$6.70^{d} \pm 0.02$
	Sig.	**	ns	**
N_{IV}	0	$12.45^{a} \pm 0.07$	$5.05^{d} \pm 0.07$	5.06 ± 0.08
	7	$17.25^{b} \pm 0.35$	$4.48^{abc} \pm 0.03$	4.33 ± 0.47
	15	$19.00^{b} \pm 0.70$	$4.87^{bcd} \pm 0.14$	4.83 ± 0.23
	30	$20.00^{b} \pm 0.70$	$4.95^{cd} \pm 0.04$	5.36 ± 0.33
	60	$52.50^{c} \pm 3.53$	$4.46^{abc} \pm 0.47$	5.98 ± 0.12
	120	$65.00^{d} \pm 3.53$	$4.36^{ab} \pm 0.13$	6.43 ± 0.39
	180	$70.00^{e} \pm 0.00$	$4.33^{a} \pm 0.17$	6.60 ± 0.12
	Sig.	**	*	ns

abc: See Table 2; ns: not significant.

Table 5
Chemical change in brine during fermentation of Geracese olives.

Olives samples	Fermentation time (days)	Titratable acidity (mEq l^{-1} brine)	pH	Salt concentration (g100 g^{-1})
G_I	0	$8.50^{a} \pm 0.00$	$4.61^{d} \pm 0.01$	$5.00^{a} \pm 0.00$
	7	$13.37^{a} \pm 1.94$	$4.54^{d} \pm 0.00$	$4.85^{a} \pm 0.00$
	15	$25.50^{b} \pm 0.70$	$4.31^{c} \pm 0.00$	$4.67^{a} \pm 0.00$
	30	$36.00^{b} \pm 5.65$	$4.02^{b} \pm 0.00$	$5.43^{b} \pm 0.17$
	60	$58.75^{c} \pm 8.83$	$3.91^{a} \pm 0.07$	$5.99^{c} \pm 0.12$
	120	$86.25^{d} \pm 5.30$	$4.02^{b} \pm 0.00$	$6.79^{d} \pm 0.14$
	180	$93.28^{d} \pm 5.27$	$3.98^{b} \pm 0.03$	$6.60^{d} \pm 0.24$
	Sig.	**	**	**
G_{II}	0	$8.50^{a} \pm 0.00$	3.97 ± 0.09	$5.00^{a} \pm 0.00$
	7	$20.62^{ab} \pm 8.30$	3.73 ± 0.02	$4.73^{a} \pm 0.17$
	15	$30.50^{b} \pm 7.77$	4.16 ± 0.61	$4.43^{a} \pm 0.60$
	30	$52.50^{c} \pm 17.67$	3.64 ± 0.02	$4.96^{a} \pm 0.00$
	60	$65.00^{c} \pm 0.00$	3.76 ± 0.05	$6.25^{b} \pm 0.16$
	120	$91.25^{d} \pm 1.76$	3.89 ± 0.04	$6.60^{b} \pm 0.08$
	180	$93.28^{d} \pm 5.27$	3.85 ± 0.02	$6.83^{b} \pm 0.08$
	Sig.	**	ns	**
G_{III}	0	$8.50^{a} \pm 0.00$	4.53 ± 0.01	$5.00^{b} \pm 0.00$
	7	$23.37^{ab} \pm 4.41$	3.73 ± 0.02	$4.61^{a} \pm 0.08$
	15	$26.75^{ab} \pm 0.35$	4.61 ± 0.02	$4.61^{a} \pm 0.08$
	30	$41.50^{ab} \pm 8.83$	4.04 ± 0.33	$5.60^{c} \pm 0.00$
	60	$58.75^{bc} \pm 22.98$	4.10 ± 0.42	$6.10^{d} \pm 0.04$
	120	$83.75^{c} \pm 22.98$	4.22 ± 0.31	$6.73^{e} \pm 0.02$
	180	$93.28^{c} \pm 17.59$	4.04 ± 0.16	$6.81^{e} \pm 0.12$
	Sig.	**	ns	**
G_{IV}	0	$8.50^{a} \pm 0.00$	$4.52^{cd} \pm 0.00$	$5.00^{b} \pm 0.00$
	7	$13.37^{a} \pm 1.94$	$4.54^{cd} \pm 0.00$	$4.85^{b} \pm 0.00$
	15	$28.00^{b} \pm 0.00$	$4.64^{d} \pm 0.09$	$4.52^{a} \pm 0.04$
	30	$45.00^{c} \pm 4.24$	$4.04^{a} \pm 0.08$	$5.38^{c} \pm 0.16$
	60	$47.50^{c} \pm 3.53$	$4.24^{abc} \pm 0.21$	$6.02^{d} \pm 0.07$
	120	$71.25^{d} \pm 1.76$	$4.37^{bcd} \pm 0.17$	$6.85^{e} \pm 0.02$
	180	$82.04^{e} \pm 0.05$	$4.14^{ab} \pm 0.07$	$6.78^{e} \pm 0.07$
	Sig.	**	**	**

abc: See Table 2; ns: not significant.

to allow the comparison among gels (data not shown). In addition, clone libraries of the partial 16S rRNA gene amplicons from brine samples were constructed (Table 6). In general is possible to assert that the dominant band 1, present in all brine samples, originated from *L. plantarum*. Moreover, the closest relative corresponding to the band 3, also detected in all brine samples, except in the control, originated to the *Leuconostoc citreum*-like sequence. In detail, profile originated from brine sample used as control (sample N_I) showed only the dominance of *L. plantarum* species, which remained stable during fermentation. Samples inoculated with starter cultures exhibited the dominance of *L. plantarum* and *L. citreum* species, which maintained the same intensity up to the end of the fermentation process. The sample inoculated with LAB starters and *L. monocytogenes* at 120th day of fermentation (sample N_{III}) and samples inoculated with *L. monocytogenes* (samples N_{IV}) taken at the different days of fermentation, considered in the present study, showed the appearance of additional amplicons which corresponded to *L. plantarum* (band 4), to *Enterococcus faecium* (bands 5 and 6), to uncultured bacterium clone (band 8 and 9) and to *Enterobacter* spp. (band 7).

When investigating bacterial population of Geracese olives during fermentation, comparison of brine samples differently treated (samples from G_I to G_{IV}) showed quite dramatic changes in the DGGE profiles with an increase in the diversity during the fermentation process (Fig. 4). Overall, samples showed some dominant bands in common, which were identified by clone libraries as *L. plantarum* (bands 1 and 2). The presence of a weak band (band 3), corresponded to *Lactobacillus coryniformis*-like sequence was also revealed. Moreover, a dominant band, which corresponded to *Lactobacillus paracollinoides* (band 6) was revealed in sample inoculated with *L. monocytogenes* from the beginning of the fermentation, while in the control and in samples G_{II} and G_{III} it appeared at 30th day of fermentation.

In detail, sample used as control (sample G_I) revealed at the beginning of the fermentation the dominance of two additional bands identified as *Pediococcus parvulus* (band 5) and *L. plantarum*

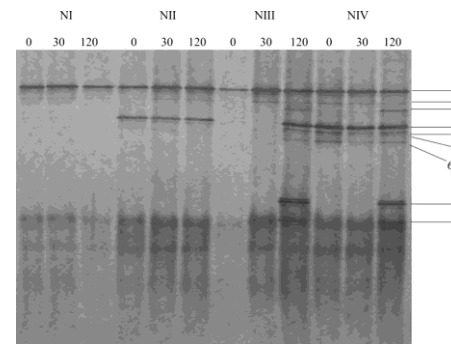

Fig. 3. DGGE profile of 16S rRNA gene of Nocellara Etnea olive samples, differently treated, taken during fermentation. Lanes 1–3: control olives at 0, 30 and 120 days of fermentation; lanes 4–6: olives inoculated with LAB starter at 0, 30 and 120 days of fermentation; lanes 7–9: olives inoculated with LAB and *Listeria* at 0, 30 and 120 days of fermentation; lanes 10–12: olives inoculated with *Listeria* at 0, 30 and 120 days of fermentation.

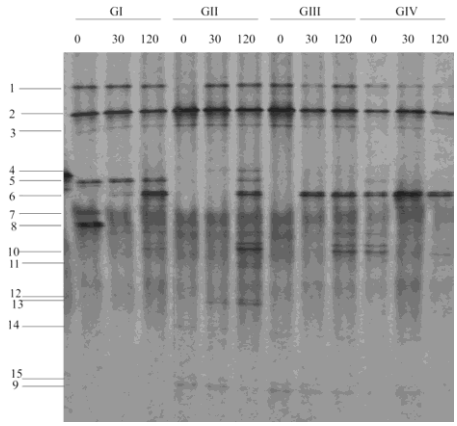

Fig. 4. DGGE profile of 16S rRNA gene of Geracese olives taken during fermentation. Lanes 1–3: control olives taken at 0, 30 and 120 days of fermentation; lanes 4–6: olives inoculated with LAB starter taken at 0, 30 and 120 days of fermentation; lanes 7–9: olives inoculated with LAB and *Listeria* taken at 0, 30 and 120 days of fermentation; 10–12: olives inoculated with *Listeria* taken at 0, 30 and 120 days of fermentation.

(band 7, Fig. 4), which disappeared during the process and the dominance of *L. brevis* species (band 4, Fig. 4) throughout the fermentation. A pronounced increase in diversity was observed in the sample inoculated with LAB starters (sample G_{II}). In fact, while at the beginning of the fermentation was detected only *L. plantarum* and *L. coryniformis* species, at the end of the process additional bands were revealed and identified as *L. brevis* (band 4), *L. paracollinoides*, (band 6), *S. thermophilus* (band 8) and *L. paracasei* (band 9). No clone was detected corresponding to weak bands (line 6, bands from 10 to 15, Fig. 4) in the clone library of G_{VI} sample at the 120th day. *L. plantarum* and *L. brevis* species dominated the whole fermentation process of samples inoculated with LAB and *L. monocytogenes* (samples G_{III}) and new un-identified amplicons (bands 10 and 11) appeared at the 120th day (line 9, Fig. 4). Any shift in the diversity was observed in sample inoculated only with *L. monocytogenes* (sample G_{IV}) which exhibited the dominance of *L. plantarum* and *L. brevis* species throughout the fermentation process (lines from 10 to 12, Fig. 4).

4. Discussion

Table olives are one of the most important fermented vegetables in the world economy. Nowadays, most table olives are produced by spontaneous fermentation (Garrido Fernández et al., 1997), in which the composition of microbial population and its dynamics are important factors influencing the final product quality. Overall, LAB are recognized to play an important role in olive fermentation and, in particular, *L. plantarum* and *Lactobacillus pentosus* are regarded as the main species used as starter culture in order to better control fermentation process (Randazzo et al., 2010).

In the present study, LAB starters were used to conduct olive fermentation and to inhibit *L. monocytogenes*, artificially inoculated onto the table olive brines. The growth/survival of the pathogen was assessed throughout a polyphasic approach, based on plating on selective medium and on culture-independent method. A previous study (Randazzo et al., 2009b) highlighted the importance of PCR-DGGE analysis for *Listeria innocua* detection in minimally processed vegetables, revealing the drawbacks of plating method. In the present study the polyphasic approach demonstrated the inability of the *L. monocytogenes* strain used to grow/survive in brine samples, which could be related to the brine conditions applied, assuring the microbiological safety of the final products. In this regard, under our conditions and by monitoring NaCl content and pH decreasing, at the beginning of fermentation, enterobacteria appear only at the beginning of the process, as autochthonous population of olive fruit, and disappeared throughout the fermentation, in accordance with previous remarks (Nychas et al., 2002; Hurtado et al., 2008).

Following microbial evolution throughout plating count, results showed yeast population is not affected by applied treatments remaining quite constant during the fermentation. It is well established that olive fermentation process results from the growth of a complex microbial population, mainly constituted by LAB and yeasts. Yeast population, ranging from 3 to 5 $\log_{10}$ cfu ml^{-1}, have been determined in brine during fermentation of different kind of olives (De Castro et al., 2002; Tassou et al., 2002). Recently, Aponte et al. (2010) identified dominant yeast species in Sicilian green table olives demonstrating their important role both during fermentation and in the final sensory characteristics of the product, in accordance to a previous work (Arroyo-López et al., 2008). It is noteworthy that, for naturally fermented olives, the main factors affecting the growth of autochthonous microbial population in the brine environment are temperature, salt concentration, nutrient availability and the presence of natural inhibitory compounds, since the fruits are not subjected to lye treatment (Tassou et al., 2002; Randazzo et al., 2011). In particular, salt concentration used following traditional procedures, varies enormously, and in general a high NaCl (around 10–12%) could affect LAB and yeast population (Bautista-Gallego et al., 2010). In the present study all samples exhibited an initial level of 5.0% NaCl, which was gradually increased during fermentation by adding coarse salt. In these conditions LAB population overcame yeasts, which were proximately 3 $\log_{10}$ cfu ml^{-1} lower than LAB, according to previous works (Panagou et al., 2008). Evaluating titratable acidity and pH changes during fermentation, GLM data showed significant differences among samples differently treated, but same trend between the two studied cultivars. In particular, Geracese brines exhibited higher acidity, lower pH values and higher autochthonous microbial population, which is probably related to the intrinsic characteristic of the cultivar. It is noteworthy that fermentation process is greatly influenced by cultivar, by phenolic compounds and their ability to diffuse outside the fruit. Phenolic compounds are essential constituents of olives, directly related to their major sensory characteristics such as flavor, astringency and colour. Most of the studied phenolic compounds exert inhibitory effect on LAB growth even if scarce information on the growth inhibition mechanism is available (Rodríguez et al., 2009). Recently, Landete et al. (2008) provided that nine of the most common olive phenolic

Table 6
Partial sequencing of the 16S rRNA gene of strains and clones from table olives.

Clone	Closest sequence relative	% identity	Accession number
GES 7	*Lactobacillus coryniformis*	99	NR02901.1
GES 6	*Lactobacillus paracollinoides*	99	NR042322.1
GET 3	*Pediococcus parvulus*	99	NR029136.1
GET 1	*Lactobacillus plantarum*	99	NR042254.1
NET 9	*Enterobacter* spp.	99	NR028912.1
NES 8	*Enterobacter faecium*	99	NR042054.1
NET 5	*Lueconostoc citreum*	99	NR041727.1

compounds did not inhibit the growth of four *L. plantarum* strains from different sources. HPLC analysis of phenol fraction of brine samples revealed that the initial oleuropein content in brines, evaluated at the 15th day of fermentation, was higher in Nocellara Etnea samples than Geracese. Nevertheless, the Nocellara samples, especially those inoculated with LAB starters, showed the highest hydroxytyrosol content. This is probably correlated to the presence of LAB starter used, which were able to accelerate debittering process influencing the permeability of olive cuticles. These results confirmed that the starter used were well adapted to the Nocellara Etnea brine conditions. Overall Geracese olives registered a higher polyphenol content than Nocellara Etnea samples, which clearly did not inhibit *Lactobacillus* population.

The diversity of bacterial population during fermentation process of Nocellara Etnea and Geracese olives was also investigated throughout PCR-DGGE analysis. Results, in accordance to those obtained by culture-dependent study, and in combination to the construction of a bacterial clone libraries, revealed the dominance of *L. plantarum*, the absence of *L. casei* in all olive samples and, for the first time, a dramatic diversity of bacterial population between the different cultivars. Results are in agreement with previous reports (Ruiz-Barba et al., 1993; G-Alegria et al., 2004; Randazzo et al., 2011), which extensively demonstrated the high versatility of *L. plantarum* species in the brine and its positive interaction with other LAB species, supporting their adaptation in the environment.

In the present work a wide biodiversity within *Lactobacillus* population was highlighted in Geracese olives, revealing, for the first time, the presence of *L. paracollinoides* and *L. coryniformis* in table olives. Both species are often found in brewery environment and are referred as beer spoilage bacteria (Suzuki et al., 2004a). In detail, *L. paracollinoides* species, which was detected as dominant species in all Geracese olive samples is a Gram-positive, non motile, non-spore forming, facultative anaerobic, catalase negative and heterofermentative bacterium, able to grow at 15 °C (Suzuki et al., 2004a). On the basis of the 16S rDNA sequence analysis and DNA–DNA hybridization, the species is closely related to *L. brevis,* (Suzuki et al., 2004b), which was also detected in Geracese olives during fermentation. These species were not revealed at the beginning of fermentation, being probably arisen from brine environment, where they were well adapted and dominated in the final product. Regarding *L. coryniformis* species, which was steadily present in all olive samples throughout the fermentation, its presence could be derived from raw vegetables, as part of the autochthonous microbial population. Besides the brewery environment, *L. coryniformis* species was also isolated from artisanal cheeses (Dolci et al., 2008), koumiss (Wu et al., 2009) and grape must and wine (Rodas et al., 2003). It was also demonstrated the probiotic attitudes of the strain *L. coryniformis* CECT 5711, able to enhance the immunity in healthy people (Olivares et al., 2006). Up to now, none information is available on the detection, by culture-dependent methods, of *L. paracollinoides* and *L. coryniformis* species in brine environment. This could be due to the inability of selective media to provide specific growth requirements and to misidentification at *Lactobacillus* species level.

In conclusion, the present study clearly demonstrated that the starter cultures used were able to properly drive the olive fermentation assuring the microbiological safety of the final product.

Acknowledgment

The authors greatly appreciated technical support of Sig. Filippo Tomaselli in the statistical analysis. Authors would also thank Giorgio Giraffa for critical reading of the manuscript.

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Part II

Lactobacillus genus: a source of probiotic bacteria

Introduction

LAB have received considerable attention over the years because they exert health promoting effects on human beings, hence the term 'probiotics' has been introduced and attributed to those microorganisms to whom a relationship between intestinal health and general well-being status has been gained. The first definition of probiotics dates back to 1965 when Lilly and Stillwell defined probiotics as "Growth promoting factors produced by microorganisms" (93). During the following years other definitions have been attributed to probiotics relating their beneficial actions to the improvement of intestinal microbial balance (132) and the modulation of mucosal and systematic immunity (118). In 2002 the FAO and WHO defined probiotics as "Live microorganisms which when administered in adequate amounts confer health benefits on the host" (49). Several species of bacteria have been proposed as probiotics and most of them belong to the genera *Lactobacillus* and *Bifidobacterium*, although *Enterococcus*, *Bacillus* and *Saccharomyces* genera harbour some probiotic strains (**Table 1**).

Lactobacillus	*Bifidobacterium*	Others
L. acidophilus	*B. adolescentis*	*Bacillus clausii*
L. brevis	*B. animalis*	*Enterococcus faecium*
L. casei	*B. bifidum*	*Leuconostoc mesenteroides*
L. curvatus	*B. breve*	*Pediococcus acidilactici*
L. fermentum	*B. infantis*	*Propionibacterium jensenii*
L. gasseri	*B. longum*	*Saccharomyces cerevisiae*
L. johnsonii		
L. reuteri		
L. rhamnosus		
L. salivarius		

Table 1. Microorganisms considered as probiotics

Selection of effective probiotics is a quite complex procedure that the joint FAO/WHO Expert Consultation has concretized in guidelines that transversally take into account (i) the origin of probiotic species isolation, (ii) their phenotypic and genetic characterization, (iii) *in vitro* tests to evaluate the probiotic features and (iv) *in vivo* studies with animal models and human clinical trials aiming to authenticate their safety and efficacy (49). The main site of health promoting action exerted by probiotics is the gastrointestinal tract (GIT), a complex ecosystem in which gut

microbiota, intestinal epithelial cells (IECs) and immune cells have evolved together establishing an alliance that results in the maintaining of gut homeostasis (109). Genetic or functional alteration of this balanced status turns into gastrointestinal disorders with different level of severity spanning from the occurrence of enteric/bacterial infections to irritable bowel syndrome (IBS) and allergic reactions (55). Probiotic microorganisms can promote a re-establishment of this broken alliance regulating the microbial homeostasis, enhancing the epithelial barrier function and activating the host adaptive immune system (144). In literature various health-promoting effects have been attributed to beneficial microbes and some of these recognized traits are listed in **Table 2**.

Health benefit	Proposed mechanisms
Alleviation of lactose intolerance	Bacterial β-galactosidase
Positive influence on intestinal flora	- Lactobacilli influence activity on overgrowth flora, decreasing toxic metabolite production - Antibacterial characterization
Prevention of intestinal tract infection	- Increase anibody production - Competitive exclusion - Gut flora alteration - Adherence to intestinal mucus preventing pathogens colonization
Improvement of the immune system	- Strengthening of non-specific defence against infection - Increased phagocytic activity of white blood cells - Increase in IgA production - Regulation of the Th1/Th2 balance, induction of cytokines production
Reduction of inflammatory or allergic reaction	- Restoration of the homeostasis of the immune system - Regulation of cytokine syntheisis
Blood lipid, heart disease	- Assimilation of cholesterol - Alteration of activity of the bile salt hydrolase enzyme
Urogenital infections	- Adhesion to urinary and vaginal tract - Competitive exclusion - Production of inhibitor compounds
Infection caused by *Helicobacter*	- Competitive exclusion - Lactic acid production - Decrease urease activity of *H. pylori* after administration of *Lactobacillus* spp.
Regulation of gut motility	- Reduced constipation

Table 2. Health promoting effects attributed to probiotics

However is important emphasize that not all probiotic microorganisms impact at the same level and with the same modality the intestinal health status suggesting that the mechanisms underlying the probiotic actions are different and overall strain-dependent (138). Although is tempting to speculate that strains belonging to the same probiotic species mediate a comparable probiotic action, scientific evidences do not support this conclusion and a generalization about probiotic efficacy cannot be done. The reason must be searched in phenotypic and genotypic variability among isolates belonging to the same well-established probiotic species (112). The natural environment where probiotics are isolated shapes the evolution and the diversity of adaptation factors leading to different survival strategies that will impact the host in different manner and consequently the diversity of probiotic factors will derive. In order to entirely disclose the variety of health promoting

effects on human host an increasing number of probiotic bacterial genomes has been sequenced and several other sequencing projects are underway (http://www.genomesonline.org) flowing together in a new discipline named 'probiogenomics'(159). Comparative genome analysis can provide the genetic basis of particular probiotic traits shared among beneficial microbes and at the same time highlights differences in them. Moreover integration of probiogenomics and functional studies with data on host gene expression in human gut can expand our understanding of the role of probiotics and their interaction with the host (74).

Probiotic lactobacilli

Lactobacilli are widely employed as probiotics in functional foods and pharmaceutical products (112). The genus *Lactobacillus* encompasses more then 100 species of Gram-positive, non-spore forming rods or coccobacilli, clustered in the subdivision of low G+C Gram-positive bacteria, and are included in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. Lactobacilli are strictly fermentative, aero tolerant or anaerobic, aciduric or acidophilic having complex nutritional requirements (76). They can be found as contaminants in a large variety of nutrient-rich environments (147) and are also natural inhabitants of human GI tract where they represent the 0.6% of the total faecal microbiota of healthy adult people (165, 166). Among the autochthonous lactobacilli species of the GI tract *L. gasseri*, *L. reuteri*, *L. crispatus*, *L. salivarius* and *L. ruminis* seem to be predominant (166), while *L. acidophilus*, *L. fermentum*, *L. casei*, *L. rhamnosus*, *L. johnsonii*, *L. plantarum*, and *L. sakei* are found at fluctuating levels (67, 166). Using culture-dependent techniques that employ enrichment, selective media and specific culture conditions lactobacilli can be isolated from faecal sample of healthy individuals (143) or from intestinal biopsies resected from terminal ileum of colonic mucosa (166). However lactobacilli seem to be naturally associated to other mucosal surfaces of human beings such as the female genitourinary tract, where the species *L. gasseri*, *L. crispatus*, *L. iners*, *L. casei*, *L. acidophilus* and *L. jensenii* represent the dominant microbiota (133). An alternative source where *Lactobacillus* species can be recovered is the oral cavity although they colonize this niche only transiently (37).
Probiotic lactobacilli are thought to play pivotal role in the maintenance and recovery of healthy state and the best results have been obtained for the treatment of several gastrointestinal disorders such as the gastroenteritis that find in rotavirus or bacteria their aetiological agents (63). The concomitant administration of antibiotics and probiotic lactobacilli results in amelioration, shortening and reduced incidence of diarrheal episodes (28). Next to that, lactobacilli have been integrated with standard application of antibiotics for the *Helicobacter pylori* (HP) eradication, a gastric infection causing ulcers and cancer in chronic inflammation. Patients suffering of HP infection show a higher rate of eradication and minor number of side effects of the antibiotics

therapy (141). Controversial results have been deduced in the treatment of IBS because the heterogeneity of the symptoms and the complexity of the pathology for which the aetiology is still unknown although some evidences suggest that the gut microbiota play a preponderant role in the development of disease (55, 75). However in IBS patients the administration of probiotic lactobacilli seems to be responsible for an improved clinical outcome counteracting inflammatory processes (75). In addition to GI applications, lactobacilli are employed in clinical trials to eradicate other infections, for instance the bacterial vaginosis caused by a depletion of indigenous *Lactobacillus* population subsequently re-established with local administration of probiotic lactobacilli integrated to antibiotic therapy against the main vaginosis-associated pathogens (36, 102). Other encouraging evidences that still need to be validated refer to the probiotic therapy for the prevention of atopic dermatitis (155) and dental caries (178).

The successful employment of lactobacilli in human clinical trials has to be searched in their high safety profile that categorized them as 'generally regarded as safe' (GRAS)(49). The joint FAO/WHO working group established the minimum tests required to characterize the GRAS status such as the determination of antibiotic resistance patterns, the assessment of specific metabolic activity, the verification of side affects during human studies and the post market epidemiological surveillance of adverse incidence in consumers. In Europe, EFSA has proposed a scheme based on the concept of "Qualified Presumption of Safety" (QPS) defined as "an assumption based on reasonable evidence" and qualified to allow specific restriction for certain applications. The QPS procedure provides a safety assessment of microorganisms evaluated according to: (i) non-ambiguous identification at the claimed taxonomic level; (ii) relationship of taxonomic identity to existing or historic nomenclature; (iii) potential pathogenicity to humans and animals; (iv) degree of familiarity with microorganism based on the weight of evidence; (v) the final use of the microorganisms (1). The correct strain identification have received particular attention because allows comparisons of potential risk with taxonomically related microorganisms, avoids the use of potential pathogens and ensures a quality control in post market surveillance of bacteremia (13). It is important emphasize that the correct taxonomic identification and the availability of proper genetics tools have other relevant implications apart of GRAS status recognition. Firstly, the possibility to estimate the amount of viable cells required to exert the health promoting action, secondly the ability to track the intestinal transit of probiotic microorganism(s) in clinical trial studies and thirdly the opportunity to choose the best carrier of probiotic administration that do not affect the beneficial properties.

Identification and typing of lactobacilli

The taxonomy of lactobacilli and other probiotic bacteria has changed significantly with the advent of genetic tools (51). Originally most of *Lactobacillus* probiotic species have been identified according to specific phenotype features such as the morphology, sugar fermentative profiles and production of specific metabolites (76). However these kinds of identifications were not enough discriminative especially when applied for identification at species, subspecies and biotype levels (21). With the advent of molecular techniques, based on the analysis of nucleic acid, the taxonomy has been revised revealing conspicuous discrepancies with the previous phenotypic identification and that was more evident for those species genetically close related where the phenotypic identification did not reflected their phylogenetic relation (30, 129, 146). Currently the most common typing methods are based on PCR molecular techniques such as PCR-DGGE/TGGE, ARDRA and RISA. However the detailed analysis of 16S rDNA as well as the 16S-23S spacer region (ITS) is still employed for identification and typing of lactobacilli by using species-specific primers because the high discriminatory capacity and for the possibility to differentiate at strain level (116, 150). Moreover based on either the genes or the ITS regions some researchers have developed multiplex PCR of species-specific primers pairs for the detection of genetically close related species (85). In the same way as oligonucleotide primers, oligonucleotide probes can also be used in hybridization experiments for species detection, identification and quantification of *Lactobacillus* species (124). In addition to 16S rDNA analysis, coding genes of highly conserved proteins such as RecA (52), GroESL (163) and elongation factor (EF) Tu (158, 160) have been used to identify lactobacilli species and to determine their phylogenetic relationships providing a comparable resolution to that of 16S rDNA gene at all taxonomic levels with a better resolution among closely related species. Recently multilocus sequencing technique (MLST) has been introduced as more robust typing method and it is based on automated DNA sequencing of alleles present at different housekeeping gene loci (99). A MLST method based on the analysis of *pgm, ddl, gyrB, purK1, gdh* and *mutS,* has been developed for the analysis of *L. plantarum* strains (39). More recently a variant of MLST, called multilocus variable-number tandem repeats (VNTR) analysis (MLVA) have applied for the fine subtyping of *L. casei*/*L. paracasei* strains (43). Pulsed-field gel electrophoresis (PFGE) that involve the digestion of genomic DNA with rare-cutting restriction enzymes and the subsequent separation of fragment in alternate reorienting electric field is still considered the gold standard technique for strain-specific identification and several protocols have been optimized for lactobacilli (152). Whole-genome sequencing and comparative genome analyses have been proposed as a tool for defining a new genomic-phylogenetic species concept for prokaryotes (115, 146). Genome technique such as comparative genome hybridization (CGH) can quickly be used to determine the genome content of bacterial strains whose genome is not known

(115) and it has already been used for comparison of members belonging to the *L. acidophilus* group with intra and interspecies diversity resolution (11). Fatty acid methyl ester (FAME) analysis has also been applied for identification of lactobacilli recovered from dairy products (57, 177). This technique is quiet cheap and useful to study the diversity of and dynamics of microbial communities, but FAME profiles are quite difficult to interpret. Identification and classification of *Lactobacillus* species can also be done by analysis of whole-cell protein patterns by SDS-PAGE where the profiles of unknown species are compared with those of known species present in a database (174).

Lactobacilli and gut ecosystem

Probiotic lactobacilli have specific targets of actions and the majority of them are localized in the human GIT that represents the largest contact area between the body and the external environments (41). In this complex ecosystem three main players are involved in the maintenance of homeostasis: the gut microbiota, the intestinal epithelium and the immune systems (109). Lactobacilli, and in general all probiotics, are thought to exert health promoting actions on human host interacting with the components of the gut ecosystem and part of the mechanisms involved have been unravelled (101), such as pathogens inhibition, via microbe-microbe interactions (136); enhancement of epithelia barrier, via microbe-intestinal epithelial cells (IECs) interactions (18) and modulation of immune response, via microbe-immune systems interactions (176). However the molecular basis driving the mechanisms are still largely unknown and the modern molecular biology based on 'omics' technologies (genomics, proteomics, metabolomics), allowing simultaneous analysis of huge numbers of genes, proteins and metabolites, have revealed insights into understanding the probiotic effectors molecules involved at each level of interaction in the gut ecosystems (17, 74). Moreover functional genomic analysis, including whole genome sequencing, genome data mining and comparative genomics, is essential to understand the cellular physiology, metabolic pathways, biosynthetic capabilities of the microorganisms and their ability to adapt to varying conditions and environments (80, 145). Thus referring to probiotic lactobacilli, it has been suggested that in addition to probiotic effector molecules, adaptation factors are essential in supporting the probiosis without being themselves health promoting (87). In lactobacilli the adaptation factors gut-associated refer to the stress resistance, metabolism flexibility, and adhesion to the intestinal mucosa. A brief description of genes and molecules responsible for adaptation factors of probiotic lactobacilli will be given below following the illustration of their unravelled health promoting mechanisms.

Adaptation factors of probiotic lactobacilli

If we think about the FAO/WHO probiotic definition, it is implicitly suggested that the beneficial microbes should arrive in the gut in a viable status in order to promote the health-promoting actions. That means that after the ingestion, probiotics must overcome several challenges such as the gastric barrier with low pH value (34) and the high concentration of bile salts secreted in the upper part of the intestine (10). The precise effects of these encountered stresses on bacterial cells are not completely understood however it can be hypothesized that the low pH level of gastric juices can acidify the intracellular compartment, reducing the enzymatic activity and damaging the DNA (154). Bile salts, that are involved in the emulsification of fats in digestion processes, affect the stability of bacterial cell membranes acting as detergents and thus having an antimicrobial activity (10). In addition to these stresses, the osmotic and the oxidative shocks are encountered as well in the GI transit (38). Genes encoding acid and bile resistance responses are essential for the tolerance of probiotics to intestinal stresses. Induction of heat shock proteins, i.e. DnaK, DnaJ, GrpE, GroES and GroEL in acid adapted cells has been shown in *L. acidophilus* CRL639 (95). Transcriptomic analysis of *L. acidophilus* NCFM has revealed the expression of stress related genes *GroEL*, *DnaK* and *ClpP* after the exposure to gastric juice following passage through an *in vivo* gastrointestinal tract model (170). Moreover in the same strain transcription of *atp* operon is induced after exposure to low acidity together with an increased activity of membrane-bound enzyme, which resulted in active expulsion of protons out of the cell and maintenance of cytoplasmic pH stable (84). Further studies have shown the presence of four loci contributing to acid resistance in the *L. acidophilus* NCFM genome. The role of the four loci in acid tolerance has been investigated by insertional mutagenesis in these regions, which resulted in acid sensitive mutants (9). The *luxS* gene in *L. rhamnosus* GG (LGG) is important for the cell survival during the GI passage being transiently up regulated after acid shock at pH 4.0 while its transcription is attenuated in acid adapted growth condition (117). In *L. reuteri* ATCC 55730, the wide genome expression analysis revealed the transcription of *clpL* gene encoding an ATPase with chaperone activity, involved in the early response to severe acid shock (164) and the same molecular chaperone is transcribed in *L. plantarum* WCFS1 in murine GI transit (22). The same strain has been deeply investigated for the bile salt tolerance in a global transcription response. Several bile-responsive gene clusters have been characterized such as the multidrug resistance (MDR) transporter to export bile, the glutathione reductase and glutamate decarboxylase involved in oxidative stresses, and genes encoding cell envelope functions responsible for maintaining the integrity and functionality of the cytoplasmic membrane including the *dlt* operon and the F_1F_0 ATPase (86). Genes encoding for bile salt hydrolases (*bsh*) have been identified in other intestinal *Lactobacillus* spp., such as *L. johnsonii* 100-100 (48) and *L. acidophilus* NCFM (107) although knockout mutants for BSH proteins did not

affect the bile tolerance and the capacity of the probiotics to survive in the GI tract, pointing out as the role of the BSH in GIT remain still elusive and should be deeply investigated.

The complete sequencing of several *Lactobacillus* spp. genomes has revealed a considerable degree of auxotrophy for amino acids and other cellular components. To compensate these auxotrophies, lactobacilli encode for multiple genes for transport and uptake of macromolecules and metabolism of complex carbohydrates (128). For instance the genome of *L. plantarum* WCFS1 encodes a large number of regulatory and transport functions, including 25 complete PTS sugar transport systems that provide a wide metabolic flexibility allowing to grow in diverse environments such as fermented foods, plants and human GIT (83). On the contrary the adaptation to a given niche specializes bacteria to grow in the presence of specific substrata and this is elucidated comparing the genome sequences of intestinal and food-adapted strains. As an example, the milk-adapted *L. helveticus* DPC 4571 posses genes for fatty acid biosynthesis and amino acid metabolism but many pseudogenes related to the utilization of several carbohydrates, while the close related gut-adapted *L. acidophilus* NCFM encodes for a conspicuous number of proteins for transport and metabolisms of a large variety of di- and polysaccharides such as raffinose and fructooligosaccharides (FOS) (4). Comparative genome analysis of LGG and *L. rhamnosus* LC705 remarks the impact of the niche on the metabolism within the same species, in fact LGG that is a gut-associated probiotic bacterium defects in the utilization of lactose on the contrary of LC705 that is a milk-adapted strain (77). However, transcription analysis is an important indicator for active metabolism of probiotics, for instance transcriptomic profiles of *L. casei* DN-114 001 in germ-free mice shows up-regulation of genes involved in carbohydrate transport and metabolism and similar results have been obtained for *L. johnsonii* NCC 533 (42, 121). The capacity to ferment sugars plays a key role in the competitive ability of lactobacilli to survive and persist in the GIT. This concept has been exploited by the application of FOS and galacto-oligosaccharides (GOS), termed prebiotics, in order to fortify the resident beneficial microbiota representing and additional carbon sources that can be metabolized by probiotics (78). However prebiotic supplement for probiotics bacteria have been questioned because some studies report that enterobacteria could also use those carbohydrates as substrate for their growth, suggesting that the symbiotic approach (probiotic in combination with prebiotic) may be not suitable or safe to treat or prevent gastro-enteric infections since pathogen's growth could be stimulated generating the opposite effect (66, 103).

Adhesion to the intestinal mucosa is a further adaptation factor that has been widely exploited in lactobacilli since the binding capacity to the mucus gel layer is thought to affect in different way the destiny of probiotics in the gut. While stress tolerance and the adaptive metabolism contribute to the survival of probiotics in the GI tract, adhesion underlies the persistence of probiotics in the gut impacting at different level the interaction with the components of this ecosystem (142). The

adhesive abilities of lactobacilli have been linked with their surface properties that are influenced by the composition, structure and organization of cell wall (19). A consistent number of adhesive molecules have been identified at cell wall including lipoteichoic acids (LTAs), polysaccharides and proteins, all of which contribute to the net physiochemical properties of the bacterial surface such as its hydrophobicity and charge (139). These binding molecules are generically termed adhesins and can be classified according to their targets in the intestinal mucosa (mucus elements, extracellular matrix), according to their localization in the bacterial surface (surface layer proteins) and according to the way they are anchored to the bacterial cell wall (sortase-dependent proteins) (157). Genes encoding mucus-binding (Mub) proteins have been found in multiple copies in different *Lactobacillus* spp. genomes (16). The predicted Mub proteins are unusually large proteins representing the largest open reading frames (ORFs) in the genome, with relatively low amino acid identity offering considerable sequence variability within surface proteins, which are supposed to have in important role in adhesion (4, 131). MUB domains have been identified in *L. reuteri* (135), in *L. johnsonii* (131), *L. acidophilus* (4) and in the dairy strains *L. helveticus* (26), indicating that their presence in not exclusive of the intestinal lactobacilli. In *L. plantarum* WCFS1, the adhesion to IECs is mediated by mannose-specific protein (Msa) and the construction of dedicated knock out mutant confirms the adhering role of the protein (130). In *L. acidophilus* NCFM fibronectin-binding protein (FbpA) and surface layer protein (SlpA) are responsible for *in vitro* adhesion to Caco-2 cells (24). LspA protein of *L. salivarius* UCC118 confers adhesive properties to Caco-2 and HT-29 cells (156). In addition to Mub proteins, *L. johnsonii* encodes for elongation factor Tu (EF-Tu) and GroEL protein responsible in mediating adhesion to Caco-2 cells (12, 60). S-layer proteins of *L. crispatus* JCM 5810 promote the binding to collagen of extracellular matrix (5). Pilin proteins encoded by *spaCBA* operon of LGG strongly contribute to the adhesion to intestinal mucus (77). LTAs of *L. reuteri* 100-23 are responsible for biofilm formation and the D-Ala mutation of LTAs affects the capacity to colonize germ free mice (167). Exopolysaccharides (EPSs) have indirect effects on the adhesion because they shield the binding molecules limiting the gut persistence. However EPSs are mainly involved in the formation of micro colonies and biofilms promoting the intercellular interactions as demonstrated for LGG (89), *L. reuteri* TMW1.106 (168) and *L. plantarum* WCFS1 (149).

In conclusion adaptation factors promote the probiotic survival during the GI transit and the persistence in the intestinal niche. Gastric barrier is the first line of defence that lactobacilli must confront while bile salts and pancreatic secretion are the second hurdles that probiotics should overcome. The rate of intestinal survival of probiotics can be measured by pharmacokinetic experiments and it has been evaluated for several lactobacilli. For instance, Collins et al. measured the survival rate in the ileum of *L. salivarius* UCC118 that was administered at $1.6x10^{10}$ cfu/gr to

volunteer and recovered in faecal samples at $2x10^6$ cfu/gr (29). *L. plantarum* NCIMB 8826 has been administered in volunteers at 10^8 cfu/gr in fermented milk and the capacity to survive in the ileum after two hour of ingestion was 10^8 cfu/ml although cell counts dropped to zero after 10 hours (161). However a prolonged intake of the strain at the same concentration estimated its survival rate around 25% but two week after the end of administration period *L. plantarum* was undetectable in the faeces (161). Persistence in gut can be evaluated by intubation at specific intestinal sites where bacteria are more likely to colonize and proliferate hence biopsy of that portion can confirm more accurately the colonization. As an example, LGG was found to adhere *in vivo* to the colonic mucosa and persist after the 12 days from administration at concentrations ranging from $6x10^1$-$4x10^4$ cfu per biopsy samples. However after 14 days the strain was undetectable in faecal sample while in the colonic mucosa was recovered up to 21 days (3). More recently a human intervention study of LGG has been done attributing to mucus-binding SpaCBA pilus the strong binding properties to intestinal mucosa and consequently its persistence in the gut (77).

Microbe-microbe interactions

The main microbe-microbe interaction investigated in probiotic lactobacilli is the antagonistic activity against entero-pathogenic bacteria responsible for gastrointestinal disorders. LAB and thus lactobacilli produce several antimicrobial compounds with broad spectrum of actions (142). Lactic acid is the prevalent organic compound resulting from the fermentative metabolism of lactobacilli and it can permeabilize the outer membrane of Gram-negative bacteria killing them (2). A part lactic acid other organic acids with none antimicrobial spectrum are produced by probiotic lactobacilli but it is interesting notice that their productions lower the local pH rendering the intestinal milieu acid creating more favourable conditions for resident microbiota instead of growth of pathogens (118). In addition to organic acids, antimicrobial substances with specific spectrum of action result from metabolism of lactobacilli such as bacteriocins (47). These compounds have been investigated as health promoting trait of probiotics because they may limit colonization of pathogens by killing them or suppressing their growth. Bacteriocins are a heterogeneous family of small, heat stable peptides with antimicrobial activity against closely related bacteria (35). *Lactobacillus salivarius* UCC118 produces antimicrobial peptide Abp118 that inhibit epithelial infection of *Listeria monocytogenes* (32). A similar inhibition of this pathogen is exhibited by plantaricin AcH produced by *L. plantarum* (14). Several studies have shown that bacteriocins produced from *L. johnsonii*, *L. rhamnosus* and *L. casei* Shirota suppress the growth of *Salmonella enterica* serovar *typhimurium* thereby preventing intestinal infection (50). Other antimicrobial molecules not completely defined are the bacteriocin-like compounds that do not fit in typical criteria defining bacteriocins and are identified on the basis of their inhibitory activity (8).

Supernatant of *L. casei* 2576 and *L. plantarum* 2142 inhibit the growth of *Salmonella enteritis* and the invasion in Caco-2 cells (119). Pathogens-probiotics interaction can be explicated by other mechanisms, such as the competitive exclusion and displacement (136). In the hypothesized mechanisms, probiotic lactobacilli are in competition for the binding sites of intestinal mucosa preventing intestinal colonization and subsequently infection by pathogens. Different strains of probiotic bacteria vary in their efficacy in blocking adhesion site for pathogens. Specific binding protein-receptor interactions and nonspecific hydrophobic group interactions have been proposed as the main mechanisms for adhesion to intestinal mucosa (120). In the first case carbohydrate moiety of mucus affects the interactions with carbohydrate binding protein while nonspecific interactions refers to the steric hindrance of binding proteins provided at intestinal cell surface. For instance in *L. helveticus* R0052 and *L. crispatus* ZJ001 the high hydrophobicity at their surface is provided by S-layers proteins and the competitive exclusion is effective against *E. coli* O157:H7 and *S. enterica* serovar *typhimurium* respectively (27, 73).

Microbe-intestinal epithelial cells interactions

In order to understand how probiotics interact with the gut epithelium a brief description of it will be provide, following the illustration of recognized probiotic-intestinal epithelial cells interactions.

The surface of the intestine is lined by columnar epithelium that is folded to form invaginations, named crypts, which are embedded in the connective tissue. Five highly specialized cell phenotypes can be distinguished in the intestinal epithelium: the fluid-transporting (or enterocytes), neuroendocrine, mucus-secreting (or globet cells), Paneth and M cells (96). All together the intestinal epithelial cells (IECs) are organized in a single layer and constitute a physical barrier that separate two different compartments, i.e. the lumen with its content and the human body. The barrier integrity is maintained by intercellular junctional complexes composed of tight junctions, adherent junctions, and desmosomes (106). Additional reinforcement to the barrier derives from the mucus gel layer that is secreted by globet cells and coats the surface of the intestine along its length excluding the Peyer's patches (92). The thickness of the mucus is variable in the different section of the GIT, ranging from 170 μm in the small intestine to 830 μm in the colon. The main constituents of mucus gel are high glycosylated proteins termed mucins that have the additional role to provide a carbon source and binding sites for the persistence of the enteric bacteria (7). The gut barrier is additionally enhanced from Paneth cells by releasing of antimicrobial substances including lysozyme, phospholipase A_2 and antimicrobial peptides (AMPs). Under physiological condition, the continual release of preformed AMPs allows chemical defence system to contribute directly to innate immunity of the crypt microenvironment by diffusing the secreted peptides into the lumen (54). A part of mucus and chemical antimicrobial compound secretion, there is a bidirectional

exchange with the gut lumen content through the M cells located in the small intestine in the follicle associate epithelium (FAE) of Peyer's patches (PP). The M cells are directly exposed to the luminal content because they are not coated from mucus and their primary function is the trans-epithelial transport of substances from the lumen to the underlying immune cells where the processing and initiation of immune responses occur (83). Enteric microorganisms, viruses, antigens and other particles can be internalized across M cells that in some circumstances are the 'Achilles heel' in the mucosal barrier because they represent the main route of access exploited by pathogens (31).

Recognized interaction between probiotic lactobacilli and IECs can be categorized in metabolic interaction and preservation of barrier integrity. Metabolism of lactobacilli has a nutritive role for enterocytes because the production of lactate from *Lactobacillus* spp. can be converted into butyric acid by other bacteria of intestinal microbiota such as *Eubacterium hallii*, providing a source of energy for IECs (45, 64). Bile salt deconjugation is another positive effect of lactobacilli metabolism on host intestinal physiology because it might affects the absorption of fats lowering the amount of cholesterol in serum. However an excessive bile salt hydrolization can negatively impact the gut health being involved in formation of gallstones (86). Probiotics preserve the barrier function by different mechanisms such as induction of mucin secretion (97), up-regulation of cytoprotective heat shock proteins (127), enhancement of tight junctions (122) and modulation of cell apoptosis (175). Secretion of mucins is driven by *MUC* gene family and in the gut MUC2, MUC3 and MUC5AC are produced by globet cells. This mechanism is dependent on adhesion to IECs as it has been demonstrated for *L. plantarum 299v*. Co-incubation of the strain with HT-29 cell line results in an increased level expression of mRNA of MUC2 and MUC3 while a spontaneous *adh* mutant, that has lost adhesive ability, does not induce mucin gene expression (97). *L. rhamnosus* GG mediates the up-regulation of MUC2 as well but further studies can highlight whether the expression of SpaCBA pili are responsible for that (105). *Lactobacillus* spp. contained in VSL#3 mixture of probiotics, increases MUC2 gene expression in LS 174T cell lines, an effect triggered by a heat-resistant soluble compound present in cell free culture supernatant (25). Heat shock proteins (Hsps) expressed by IECs are thought to stabilized the cellular cytoskeleton of IECs rearranging the actin filament by cross-linking at the apical epithelial surface that is one of the site entry disrupted after entero-pathogenic invasion (171). Hsps that cooperate in the formation and function of the eukaryotic cell cytoskeleton are Hsp60, Hsp70, Hsp90 and Hsp100 (90). It has been proposed the probiotic bacteria can stimulate IECs to produce Hsps reinforcing the barrier integrity. Invading experiment, in which intestinal model cell lines have been infected by *Salmonella* spp. and then co-incubated with *L. casei*, reveals the expression of Hsp70 *via* stabilization of the apical cytoskeleton preventing membrane ruffling and thus impeding the invasion (100). *L. acidophilus* LB antagonized the Caco-2 cytoskeleton rearrangement by invasive *E. coli*, avoiding the formation

of brush border lesions promoted by the pathogen (91). In the spent culture supernatant (SCS) of LGG, an acid and heat stable low molecular weight peptide induces in time- and concentration-dependent manner the expression of Hsp25 and Hsp72 that seem to protect IECs from oxidative stress, perhaps preserving the barrier integrity (151). Several studies have reported that invasion of pathogens results in increased paracellular permeability altering the function of interepithelial tight junction proteins, i.e. occludin, claudin and junctional adhesion protein. Normally occludin and claudin are associated with cytosolic proteins named zonula-occludin proteins ZO-1, ZO-2 and ZO-3 forming cytoplasmic plaques (59). Invasion of IECs from entero-pathogens disassembles ZO proteins from occludin and claudin increasing paracellular permeability and translocation of pathogens in lamina propria (169). Gene expression studies have demonstrated that *L. plantarum* MB452 alters expression levels of numerous tight junction-related genes, including those encoding occludin and cytoskeleton anchoring proteins. *L. acidophilus* increases transepithelial resistance (TER) of HT-29 and Caco-2 cells by augmenting levels of phosphorylation in occludin proteins (98). In polarized monolayer Caco-2 cells, the reduced TER caused by *L. monocytogenes* invasion increases after co-incubation with *L. plantarum* MF1289 and *L. salivarius* DC5 (81). In human colon crypt-like T84 cells, *L. casei* DN-114 001 is able to abrogate in a dose dependent-manner the paracellular permeability and redistribution of ZO-1 induced by *E. coli* EPEC (123). *L. rhamnosus* GG antagonizes the EHEC-induced changes in paracellular permeability in T84 epithelial cells, affecting the TER and expression of claudin and ZO-1 (72). In addition to their effects on tight junction proteins, probiotics are able to prevent cytokine- and oxidant- induced epithelial damage by promoting cell survival. For instance soluble factor p75 and p40 released from LGG prevent epithelial cell apoptosis through activating anti-apoptotic Akt in P13k-dependent manner and inhibiting pro-apoptotic p38/MAPK (mitogen-activated protein kinase) (175). Moreover these two proteins are also able to reduce the injuries caused by tumour necrosis factor alpha (TNF-α) in murin colon tissue explants and inhibit TNF-α-induced apoptosis in MCE cell line (137).

Microbe-immune system interactions

Intimate connected to IECs, the intestinal immune system organ is continuously exposed to the luminal content containing microbial antigens that derive from the intestinal colonization. The stimuli provided by colonization of commensal bacteria are essential for the development of a fully functional and balanced immune system, including not only the production of secretory IgA (sIgA) that contribute to a specific immunity against invading pathogenic microorganisms, but also the induction of tolerance toward innocuous food and bacterial antigens (20). In the GIT the immune system is organized in gut-associated lymphoid tissue (GALT) that is composed of effector sites,

including the intra-epithelial lymphocytes (IEL) and lamina propria (LP), and inductive sites such as mesenteric lymphoid nodes (MLN) and Peyer's patches (PP) (**Figure 1**) (6).

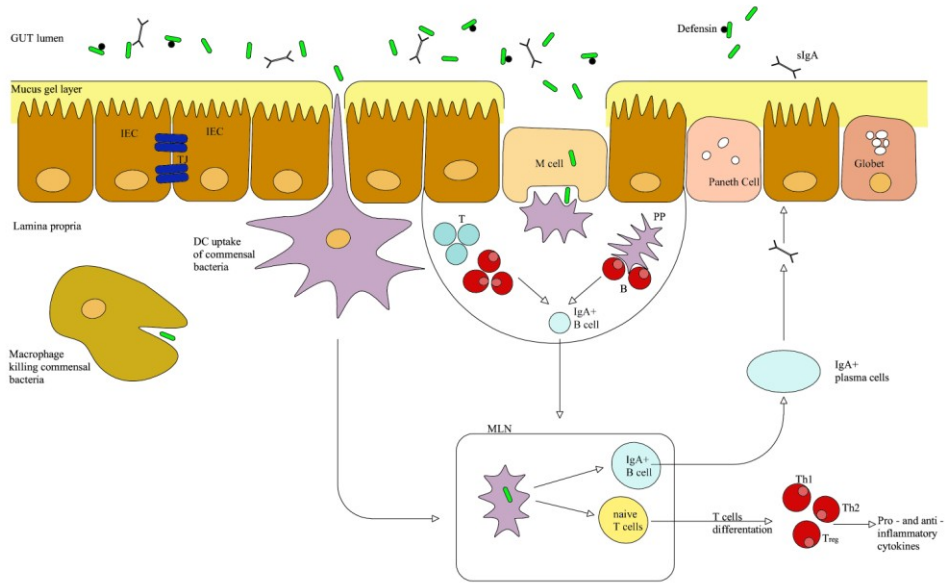

Figure 1. Schematic representation of GALT and immune responses mediated by dendritic cells. More detailed explanation can be found in the main text.

Each site contains immunocompetent cells such as phagocytic cells (neutrophils, monocytes and macrophages) and natural killer cells (NKs) that participate to the innate immunity providing to the host the first line of defence against infectious agents (44). The second immune defence line is provided by dentritic cells (DCs) that are located in LP and in the dome area of PP acting as antigen-presenting cells (APCs) thus initiating the adaptive immune response through the production of cytokines (140). DCs can capture antigens and bacteria (included probiotics) from lumen by extruding dendrites between IECs without disrupting the integrity of epithelium barrier or alternatively can take up antigens and/or bacteria internalized by M cells that are located in follicle-associated epithelium (FAE) overlying the PP (134). Recognition from DCs of microbial products is mediated by pathogen recognition receptors (PRRs) that are also expressed in macrophages and IECs (110). At this stage DCs initiate the immune responses migrating in MLN where naïve T cells are, driving their polarization in T helper (Th1, Th2 or Th3) or T regulatory cells (T_{reg}) according to the antigen presented (33). Both type of polarized T helper cells produce cytokines, Th1 cells produce pro-inflammatory cytokines such as IFNγ, TNFα and IL-2 that stimulate phagocytosis

while Th2 cells produce the cytokines IL-4, IL-5, IL-6 and IL-13 that induce humoural immunity by secretion of IgA. T_{reg} cells, that produce IL-10 and TGF-β cytokines, have been proposed to induce oral tolerance, suppress allergies and asthma and induce tolerance to commensal bacteria, included probiotics (6). It has been shown *in vitro* that the exposure of DCs to a selection of probiotics can instruct DCs to drive T_{reg} to produce IL-10 whose production is typically measured because is an anti-inflammatory cytokine that suppresses IL-12 and IFNγ production (33). Moreover IL-10 down regulates antigen presentation and inhibits macrophages activation with resulting lower level of pro-inflammatory cytokines. In addition to IL-10, IL-12 production is commonly measured as well after co-incubation of DCs with probiotic lactobacilli because it is associated with the polarization of T cells into Th1 with increased level of IFNγ (172). In some cases probiotic lactobacilli can also induce high level of IL-12 thus it has been suggested that the ratio of IL-10/IL-12 and IL-10/ TNFα should be taken into account. In a recent study of immunomodulatory properties of 42 strains of *L. plantarum*, a comparison of IL-10 and IL-12 ratios reveals as the level of these cytokines can vary independently of each other distinguishing strains with pro-inflammatory and anti-inflammatory properties (111). Other example of anti-inflammatory cytokine production with primed Th2 and T_{reg} response can be done, for instance the treatment of DCs with probiotic mixture of VSL#3 containing three species of *Lactobacillus* spp., reduces production of IFNγ by DC-stimulated T cell being related to a decreased number of Th1 cells (65). Similarly, co-incubation of DCs with *L. paracasei* B21060 results in lower level of IFNγ, IL-2, IL6 and IL-10 suggesting a reduced Th1 cell population (113). In intestinal inflammation caused by *Helicobacter hepaticus* in IL-10 knock-out mouse, the level of IL-12 and TNFα decreased after administration of *L. paracasei* 1062 and *L. reuteri* 6798 (94, 125). DCs exposed to *L. reuteri* 100-23 and then co-cultured with MLN cells showed an increased number of $FOXP3^+$ T cells (suppressor of T cells) along with the concomitant reduction of T cell proliferation and enhanced T_{reg} population, suggesting that lactobacilli can influence this cell population exerting anti-inflammatory effects (94).

Epithelial crosstalk

Immune responses are activated by IECs as well that represent the highest surface exposed to commensal bacteria and probiotics in GIT. A crucial factor in recognition of lactobacilli is the expression from IECs of PRRs activated by microorganism-associated molecular patterns (MAMPs), which are widespread and conserved among microorganisms, often located on bacterial cell surface and not expressed by the host (88). The best-characterized signalling receptors are Toll-like receptors (TLRs) that are transmembrane proteins located at cell surface, in intracellular compartment or in the cytosol (79). In addition to TLRs family, extra cellular C type lectin receptors

(CLRs) and intracellular nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs) are known to transmit signal on interaction with bacteria **(Table 3)** (62, 148).

PRR	Cellular Localization	MAMP	Origin of MAMP
TLR2	Surface	Lipopeptides, Lipoproteins, LTA	Bacteria
TLR2/TRL1	Surface	Triacylated lipopeptides	Gram-
TLR2/TLR6	Surface	Diacylated lipopeptides	Gram+
TLR3	Intracellular compartment	dsRNA	Virus
TLR4/MD2	Surface	LPS	Gram-
TLR5	Surface	Flagellin protein	Bacteria
TLR7	Intracellular compartment	ssRNA	Virus
TLR8	Intracellular compartment	ssRNA	Virus
TLR9	Intracellular compartment	DNA	DNA virus, bacteria
TLR11	Surface	Uropathogenic bacterial components	Uropathogenic bacteria
NOD1	Cytoplasm	Meso-DAP	Gram- PG
NOD2	Cytoplasm	MDP	Gram+ PG

Table 3. PRRs localization, MAMPs and their origin. Adapted from (172)

The interaction between PRRs-MAMPs involve recruitment of adaptor molecules, such as myeloid differentiation primary response gene 88 (MyD88) that in turn activate MAPK pathway and the nuclear factor *k*B (NF-*k*B) pathway signalling cascade. Normally, in an inactivated state NF-*k*B is located in the cytosol as protein complex with the inhibitory protein I*k*Bα but TLR and NLR signalling leads to the phosphorylation of I*k*Bα, its ubiquitination and degradation by the cell proteasome (69). Liberated NF-*k*B is then translocated into the nucleus and induces the transcription of specific genes that will drive production of a broad range of chemokines and cytokines, TNF-α, growth factors and inducible beta-defensins (BDs) (**Figure 2**) (173). It has been observed that the main cytokine produced by IECs from activation of NF-*k*B pathway is IL-8 that functions primarily as neutrophil chemo-attractant. Probiotics can prevent NF-*k*B signalling and influence the IL-8 downstream secretion. For example Zhang et al. investigated the effects of LGG exposure to epithelial cell model, demonstrating the ability of this strain to decrease the I*k*Bα degradation, resulting in reduced level of TNF-induced IL-8 production (179). Pre-treatment of epithelial cells with *L. casei* DN-114 001 decreases *Shigella flexneri*-induced NF-*k*B activation due to inhibition of I*k*Bα degradation (153). Comparable results have been obtained for *L. reuteri* by using T84 and HT-

29 cell line in which the anti-inflammatory effect is related to the diminished IL-8 production (153). However probiotic lactobacilli differ in their capacity to augment IL-8 expression and some of them seem to rather increase epithelial cell production of this interleukin as in the case of *L. plantarum* 299v in HT-29 epithelial cell model (108). A part IL-8 production, enterocytes can be a source of other cytokines such as IL-6 that is a multifunctional cytokine involved in diverse biological processes such as host response to enteric pathogens, acute-phase reaction and clonal expansion of B cells triggered to produce IgA (162). Co-incubation of murine primary intestinal epithelial cells with *L. casei* CRL 431 and *L. helveticus* R389 increased the level of IL-6 production together with the number of IgA^+ cells in the intestinal lamina propria without affecting the recruitment of $CD4^+$ T_{reg} population after the oral administration of these bacteria, suggesting that the immune responses initiate prior to encounter immunocompetent cells (40).

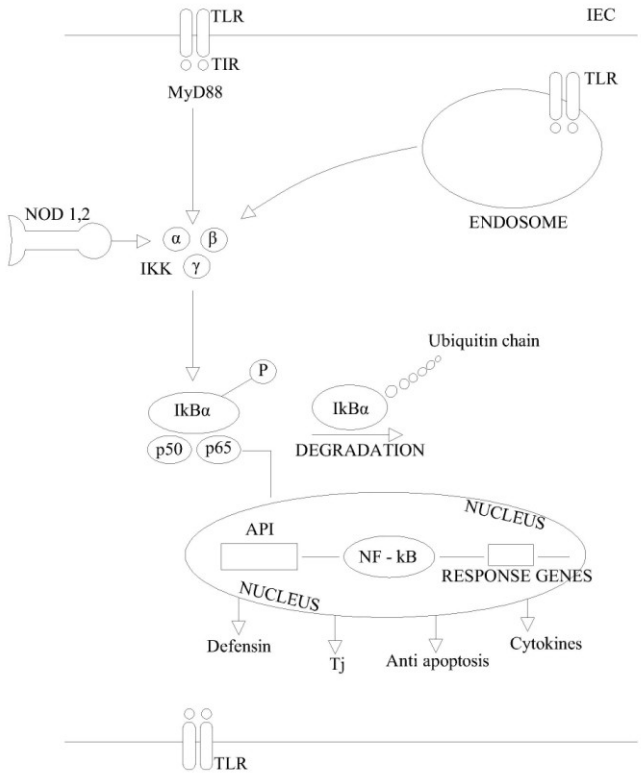

Figure 2. IEC and NF-kB pathway. Adapted from (88, 173)

Although the aforementioned studies demonstrate the involvement of IECs in activation immune responses, few MAMPs and the related PRRs have been identified for probiotic lactobacilli (23). It has been demonstrated that bacterial cell wall compounds can signal mainly through binding TLR2 in combination with TLR6. More then ten years ago bacterial lipoproteins were shown to recognize

TLR2 and crystallographic structural data revealed that the lipid chains bind the in a hydrophobic pocket in the extracellular domain of TLR2 (71). In addition to lipoproteins, other bacterial cell wall compounds of Gram-positive act as ligands for TLR2 such as LTAs (46). Recently the role of D-alanine substitution of the polyglycerol backbone of LTA has been investigated for modulation of specific immune responses in *L. plantarum* NCIMB8826. Construction of *dlt* mutant that incorporate less D-Ala in its LTA impacted significantly on the immunomodulatory properties of the bacterium showing a consistent reduction of pro-inflammatory cytokine production by peripheral blood mononuclear cells (PBMCs) when compared to the wild type strain immune stimulation (61). In contrast a *dltD* mutation in LGG did not alter the cytokines production by intestinal cells in comparison to the wild type strain but the *dlt* mutant was more sensible to anionic detergent and the strain increased the rate of autolysis (126). Recently deletion of LTA in *L. acidophilus* NCK56 was observed to down regulate IL-12 and TNF-α production in DCs with a concomitant increased level of IL-10 responsible for suppression of T cell proliferation (114). However peptidoglycan fragments of Gram-positive can also trigger immune response via TLR2 although recent studies revealed the participation of intracellular receptor NOD2 that recognizes the muramyl dipeptide (MDP) present in all lactobacilli (58). For instance the peptidoglycan fragments of *L. rhamnosus* Lr32 and *L. salivarius* Lr33 trigger DCs responses and T cell polarization in NOD2-dependent way although it cannot be excluded that TLR2 cooperates in signalling (53). Intracellular component of lactobacilli are also ligand for PRRs and specifically the methylated cytosin-guanodin dinucleotides (CpG) motif of DNA (68). Pre-treatment of HT-29 cell line with DNA from probiotic cocktail VSL#3 delays the NF-kB activation and attenuates the secretion level of IL-8 in response to *Salmonella* DNA and similar trend is observed in T84 epithelial cells treated with DNA from LGG (56, 70). Exopolysaccharides (EPSs) of lactobacilli can be putative ligands for PRRs although it remains to be established which receptors can mediate the immune responses. However for some strains it has been reported that they can be responsible for cytokine production, for example EPSs from *L. rhamnosus* RW-9595 M stimulate production of IL-6 and IL-12 in PBMCs and macrophages (15). EPSs of *L. casei* Shirota suppress cytokine production in macrophages suggesting that the capsular polysaccharide can act as immune modulator reducing an excessive response during activation of macrophages (104). Extracellular proteins secreted by lactobacilli can modulate the activity of immune cells. S-layer protein A (SlpA) released from *L. acidophilus* NCFM has been shown to bind the surface lectin receptor DC-SIGN of DCs inducing IL-10 secretion and inhibiting T cell proliferation (82).

In conclusion recent functional analyses and molecular studies have identified some of the genes and molecules offering the health benefit of probiotics on human host revealing a close interaction with all components of GI-ecosystem. Different genes have been recognized to be involved in

mucin secretion, in regulation of the different signalling pathways resulting in pro- and anti-inflammatory effects and strengthening the epithelial tight junctions, which have a protective role on intestinal epithelial barrier functionality. However only a limited number of genes have been identified in this regard and additional studies are necessary to uncover all genes involved and to clarify the specific mechanisms at the molecular level. Moreover considering the biodiversity of probiotic *Lactobacillus* spp. and the fact that their mode of action is species and even strain dependent more stringent criteria should be taken into account for selection of new candidate probiotic bacteria. The possibility to use several intestinal cell lines and immune cells are a valid instrument to simulate *in vitro* the host-microbe interactions and collect evidences of probiosis but reproducible results should be observed *in vivo* in properly conducted clinical studies (such as randomized double-blind trials). This will allow to establish the right employment of probiotics in the treatment of some gastrointestinal disorders and the development of new pharmaceutical products or functional foods.

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Aim of the study

The aim of the study presented in the second part of this thesis was to investigate the variability of different adaptation factors and health promoting effects of *L. rhamnosus* strains recovered from different ecological niches. The strains have been analyses by comparative analysis at genotypic and phenotypic level with the further task to understand the ecological versatility of *L. rhamnosus* species. Moreover considering that *L. rhamnosus* strain GG and *L. casei* are two species widely marketed as probiotics, a comparative analysis of some health-promoting traits will be provided in order to highlight differences in their claimed beneficial effects.

Comparative Genomic and Functional Analysis of *Lactobacillus casei* and *rhamnosus* Strains Marketed as Probiotics

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Running title: Characterization of probiotic-marketed strains

Keywords: *L. rhamnosus*, *L. casei*, adhesion, genome, pili

-Submitted to Applied Environmental Microbiology-

Abstract

Four *Lactobacillus* strains were isolated from marketed probiotic products, including *L. rhamnosus* strains from Vifit (Friesland Campina) and Idoform (Ferrosan), and *L.casei* strains from Actimel (Danone) and Yakult (Yakult Honsa Co.), respectively. Their genomes and phenotypes were characterized and compared in detail with *L. casei* strain BL23 and *L. rhamnosus* strain GG. Phenotypic analysis of the new isolates indicated differences in carbohydrate utilization between *L. casei* and *L. rhamnosus* strains, which could be linked to their genotypes. The two isolated *L. rhamnosus* strains had virtually identical genomes to *L. rhamnosus* GG, testifying for their genomic stability in products. The *L. casei* strains showed much greater genomic heterogeneity. Remarkably, all strains contained an intact SpaCBA pili gene cluster. However, only the *L. rhamnosus* strains produced mucus-binding SpaCBA pili. Transcription initiation mapping demonstrated the insertion of an *iso-IS*30 element upstream of the pili gene cluster in *L. rhamnosus* strains but absent in *L. casei* strains had constituted a functional promoter driving the pili gene expression. Remarkably, all *L. rhamnosus* strains triggered an NF-κB response *via* the TLR-2 receptor in a reporter cell line, whereas the *L. casei* strains did not or to a much smaller extent. This study demonstrates that the two *L. rhamnosus* strains isolated from probiotic products are virtually identical to *L. rhamnosus* GG and further highlights the differences between these and *L. casei* strains marketed widely as probiotics, in terms of genome-content, mucus-binding and metabolic capacity, and host signalling capabilities.

Introduction

Lactic acid bacteria (LAB) are a phylogenetically related group of Gram-positive bacteria sharing as common metabolic property, the production of lactic acid as main end product of carbohydrate utilization (1). Many LAB are traditionally used as culture starters in industrial dairy fermentations of raw materials, such as milk, vegetables and meat. However, in recent years, specific LAB strains have been associated with health benefits and are marketed as probiotics in a highly successful way, reaching market volumes of over 100 B$ (2, 3). Most of these marketed strains belong to the genus *Lactobacillus* that represents the largest group of LAB, encompassing more than 100 cultivable bacterial species (4). They are found in a large variety of food-related habitats and naturally associated with mucosal surfaces such as oral cavity, vagina and gastrointestinal (GI) tract.

Currently, strains belonging to the following species *L. acidophilus, L. plantarum, L. johnsonii, L. reuteri, L. paracasei, L. casei* and *L. rhamnosus* play a predominant role in the probiotics market where they are known under proprietary brand names (5). Many of these LAB strains marketed as probiotics were selected according to their *in vitro* abilities to endure to the harsh physical-chemical environment of the human GI tract, *i.e.* low pH, high concentration of bile salts and, also for their remarkable adhesive properties to human mucus and anti-pathogenic activity (6). To demonstrate their health-promoting abilities, a number of *Lactobacillus* strains, including *L. rhamnosus* GG, have been successfully used in human interventions with subjects suffering from GI disorders and atopic dermatitis (7, 8). Comparative studies have shown that the probiotic features and their associated health properties are strain-specific and cannot be generalized, indicating that it is essential to characterize the *Lactobacillus* strains at the genome level, as it has been done for a limited number of paradigm probiotics (3). This has promoted rapid insights into the diversity, evolution and molecular basis

underlying health benefits of these strains, resu lting in a research area that has been termed probiogenomics (9). One of the most studied and widely marketed probiotic strain is the human-isolate *L. rhamnosus* GG (commercialized under the name LGG). We have recently characterized this and another *L. rhamnosus* strain LC705 at the genomic and phenotypic level (10). This analysis has identified candidate genes contributing to its adaptability in the intestinal tract and the construction of dedicated knock-out mutants contributed to establishing detailed gene-function relationships. Thus specific surface macromolecules and their role in gastrointestinal fitness of *Lactobacillus* GG have been characterized. For instance the long galactose-rich exopolysaccharide (EPS) molecules form a protective shield against antimicrobial peptides secreted by intestinal epithelial cells, promoting the survival of *L. rhamnosus* GG in the intestinal tract (11). In addition, *L. rhamnosus* produces two secreted proteins, p75 and p40, reported to signal to the MAPK pathway in intestinal cells (12) that recently have found to be the glycosylated D-glutamyl-L-lysyl endopeptidase Msp1 and an essential cell wall hydrolase Msp2, respectively (13, 14). Moreover, several surface proteins have been investigated because they mediate the interaction with human host, including the mucus-binding factor MBF (15) and the highly repeated protein MabA that appears to contribute to biofilm formation (16). However, a major driver of adhesion to intestinal mucosa and biofilm formation are the mucus-binding pili of *L. rhamnosus* GG encoded by the *spaCBA-strC* gene cluster (10, 17, 18). These pili are protruding protein fibers consisting of multimers of SpaA, decorated by the mucus-binding proteins SpaC and covalently linked to the peptidoglycan by the product of *spaB* (17). Comparative genome analysis has shown that *L. rhamnosus* and *L. casei* genomes are highly related (4). This is illustrated by the observation that not only *L. rhamnosus* but also *L .casei* strains produce the highly identical Msp1 (p75) and Msp2 (p40) proteins that have similar function in both species (19, 20). However, in spite of the fact that strains of *L. casei* are widely marketed as probiotics (5), the genomes of many commercial

L. casei strains have yet to be reported. Currently, the complete genome sequences of strains *L. casei* ATCC 334 (21), *L. casei* BL 23 (22), *L. casei* Zhang (23), *L. casei* LCW2 (24) and *L. casei* BD-II (25) are available and some have been subject to detailed comparative genome analysis (26, 27). However, only a very limited number of functional studies have been reported. The best characterized strain is *L. casei* BL23 used in studies that indicated anti-inflammatory properties in an animal model of intestinal inflammation (28) and the capacity to bind extracellular matrix proteins (fibronectin and collagen) ascribed to the FbpA surface and other proteins that are also partly conserved in *L. rhamnosus* GG (29). Other documented properties of *L. casei* species relate to its resistance to the stresses encountered during the gastrointestinal passage mainly due to the acid and bile tolerance (30-33).

The aim of the present study is to provide a comparative analysis of widely marketed probiotic *Lactobacillus* strains belonging to *L. casei* and *L. rhamnosus* species. Hence, *L. rhamnosus* strains were isolated from the commercial products Vifit and Idoform, while *L. casei* strains were isolated from products branded as Yakult and Actimel. These were characterized at genotypic and phenotypic level for their carbohydrate metabolism, adhesive and immumodulatory properties. The validity of this approach was confirmed by the high identity to reported *L. rhamnosus* GG genome of the *L. rhamnosus* re-isolates from commercial products, testifying for the product stability of this widely used probiotic strain.

Materials and Methods

Isolation of Strains, Growth Condition and DNA extraction. The bacterial strains used in this study are listed in Table 1. *L. rhamnosus* GG (ATCC 53103) was obtained from Valio culture collection (Valio Ltd, Helsinki, Finland) and *L. casei* BL23 (ATCC 393) that was cured from its lactose plasmid pLZ15 was kindly provided by Institute of Agro chemistry and Food Technology (Valencia, Spain). *L. rhamnosus* strains were isolated as dominant population from food and pharmaceutical products commercialized as carrying *L. rhamnosus* GG under brand names Vifit (Friesland Campina, The Netherlands) and Idoform (Ferrosan, Denmark), resulting in *L. rhamnosus* strains LrV and LrI, respectively. *L. casei* strains were derived from the food drinks branded as Yakult (Yakult Honsha Co., Japan) and Actimel (Danone, France) and were termed *L. casei* strains LcY and LcA, respectively. The isolation of the strains LrV, LcY and LcA was carried out by homogenizing 1 mL of product in 9 mL of sterile PBS while one Idoform tablet was dissolved in 10 mL of sterile PBS to isolate LrI. The isolation was realized by generating single colonies *via* serial dilution and plating on MRS broth (Difco BD, NJ,USA) solidified with 1 % w/v agar plates incubated anaerobically at 37°C for 48h. Colonies of each product were selected, inoculated in MRS broth and propagated overnight anaerobically at 37°C. From each bacterial culture, an aliquot was used for chromosomal DNA extraction using Wizard Genomic DNA Purification Kit (Promega, WI, USA) following the manufacturer's instructions.

Molecular Typing. The identification at the species level of bacterial isolates was performed by amplification of *tuf* gene as described previously (34). Briefly, *tuf* gene was amplified by PCR using 10 mM Tris-HCl, 50 mM KCl, 1.5 mM$MgCl_2$, 200 µM of each dNTPs (Finnzymes, Finland), 10 pmol of PAR primer (5'-GACGGTTAAGATTGGTGAC-3'), CAS primer (5'-ACTGAAGGCGACAAGGA-3') and RHA primer (5'-GCGTCAGGTTGGTGTTG-3'), 50 pmol of CPR primer (5'-

CAANTGGATNGAACCTGGCTTT-3'), 25 ng of genomic DNA and 2.5 U of Dynazyme DNA polymerase (Finnzymes, Finland) in a final volume of 50 µL. Multiplex PCR assays were run in a DNA Engine Peltier Thermal Cycler (Biorad, CA, USA). Amplification products were resolved by DNA gel electrophoresis (Sigma, MO, USA) and gel was stained by ethidium bromide.

Fermentative Profiling. The sugar degradation and other catabolic properties of the *Lactobacillus* strains were characterized using API CH 50 kit (Bio-Merieux, Marcy L'Etoile, France). All strains were grown until logarithmic phase and then inoculated in API galleries as per the manufacturer's instructions. API galleries were incubated at 37° C for 48 h prior to colorimetric analysis.

Human Mucus Binding Assay. Adhesion assays of the *Lactobacillus* strains radiolabelled by ^{3}H-thymidine were performed as described previously (35). In brief, Maxisorp microtiter plates (Nunc, Denmark) were coated with 100 µL of mucus solution in PBS at final concentration of 0.5 mg/mL and incubated overnight at 4°C. The wells were washed with PBS to remove unbound mucus and 100 µL of ^{3}H-thymidine radiolabeled bacterial suspensions at OD_{600} = 0.25±0.01 were added. The microtiter plate was incubated at 37°C for 1h. Next, wells were washed with PBS to remove unbound bacteria and incubated at 60°C for 1h with 1% w/v SDS-0.1 M NaOH solution. The radioactivity of lysed bacterial suspensions was measured by liquid scintillation counting (Wallac 1480 WIZARD 3 automatic gamma counter). The percentage ratio between radioactivity values of bound bacterial suspension and total bacterial suspension initially added to the well, measured the adhesion to human intestinal mucus. For each strain, binding assay was performed at least in triplicate. Antiserum-mediated mucus binding assay was also performed for GG, LrV and LrI in the presence of polyclonal SpaC antiserum exactly as described previously (10). Similar procedure mentioned above was subsequently performed and radiolabeled bacteria were added to intestinal immobilized mucus upon incubation with 1:100 SpaC immune serum.

148 **Bile Salt Sensitivity**. All strains were propagated in MRS broth at 37°C anaerobically. Next, the
149 bacterial suspensions were adjusted to OD_{600} = 1.5 and further diluted in sterile PBS. Three microliters
150 of samples were spotted on MRS agar plates supplemented with 0.5 % w/v Ox gall bile salts (Sigma,
151 MO, USA). Plates were then incubated for 48 h at 37°C in anaerobic conditions prior to visual
152 examination.

153 **Western Blotting Analysis of Cell Wall Proteins.** Bacterial suspensions (OD_{600} = 1) were used to
154 extract cell wall-associated proteins from the *Lactobacillus* strains. Cell pellets were washed once with
155 PBS and disrupted mechanically by bead-beating with sterile quartz beads (Merck, Germany). Cell
156 wall fraction was resuspended in 500 µL of PBS, pelleted at high speed for 30 min at 4°C and
157 subsequently digested for 3h at 37°C in a 50 µL lysis buffer containing 50mM Tris-HCl, 5mM $MgCl_2$,
158 5mM $CaCl_2$, 10mg/mL lysozyme and 150 U/mL mutanolysin. Samples were mixed with 12.5 µL of 4X
159 Laemmli buffer (BioRad, CA, USA) and denatured at 99°C for 10 min. Cell-wall associated proteins
160 were separated by SDS-PAGE using a 10% v/v polyacrylamide gel and then electroblotted onto 0.2 µm
161 nitrocellulose membrane (BioRad, CA, USA). Polyclonal rabbit SpaA antiserum (1:10,000 dilution)
162 (17) and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, UK) (1:10,000) were
163 used as a primary and secondary antibody in 5% non-fat milk/PBS, respectively. Membranes were
164 blocked with 5% non-fat milk/PBS, and washed with 0.05% Tween® 20–PBS between incubations.
165 Bands were visualized by using chemiluminescence following specifications of the supplier (Western
166 Lightning Chemiluninescence Reagent Plus, Perkin Elmer, UK).

167 **TLR Response Assay.** HEK-blue™ hTLR2, HEK-blue™ hTLR4 and HEK-blue™ hTLR5 cell lines
168 (Invivogen, CA, USA) which constitutively express the TLR receptor and an alkaline phoshatase gene
169 fused to the NF-κB gene, were used in these assays. All cell lines were grown and subcultured at 70-80%

confluency in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 4.5 g/L D-glucose, 50 U/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL µg/mL Nor mocin™, 2 mM L-glutamine, 10% v/v of heat-inactivated foetal bovine serum (Integro BV, The Netherlands). For each cell line, the immune response experiment was carried out splitting HEK-blue™ cells in flat-bottom 96-well plates and stimulating them by addition of bacterial suspension adjusted to OD_{600} = 0.1 or TLR-specific ligands. The 96-well plates were incubated for 24 h at 37°C in 5% CO_2 incubator. Receptor ligands as PAM(3)CSK(4) (1 ng/mL for hTLR2), LPS-EB (1 ng/mL for hTLR4), and recFLA-ST (10ng/mL for hTLR5) were used as positive control while maintenance medium without any selective antibiotics was used as negative control. The activity of the secreted alkaline phosphatase, the product of the reporter gene fused to the NF-κB gene, was determined by incubating 20 µL samples of the reporter cell line supernatant with 180 µL of QUANTI-Blue™ (Invivogen, CA, USA) at 37°C followed by measuring the OD_{620} at after incubation with controls or *Lactobacillus* cells for 1 h, 2 h and 3 h. All assays were performed in triplicate for each sample.

Immuno-Electron Microscopy. *Lactobacillus* strains were grown to stationary phase and then used for transmission electron microscopy analyses. Sample preparation was done according to immunogold-labeling protocol described previously (17). Briefly, drops of MRS-grown cultures were incubated on Formvar carbon-coated copper grids for 30 min at room temperature. Grids were washed three times with 0.02 M glycine in PBS and then incubated 15 min with blocking solution of 1% w/v of bovin serum albumin (BSA). Next, polyclonal SpaA antibody was diluted 1:100 in 1% BSA, in which the grids were incubated for 1h, then washed with 0.1% BSA and incubated for 20 min with protein A gold conjugates (10 nm diameter). Grids were then washed several times in PBS, fixed for 5 min using 1% glutaraldehyde, washed again with MilliQ distilled water and stained with 1.8% methycellulose-0.4%

192 uranyl acetate solution. Grid visualization was carried out using JEOL 1200 EX II transmission
193 electron microscope (JEOL Ltd., Japan).

194 **Genome Sequencing and Bioinformatic Analysis of *L. rhamnosus* and *L. casei* strains**. Genomic
195 DNA of the *L. rhamnosus* strains LrV and LrI and the *L. casei* strains LcA and LcY were sequenced
196 on a SOLiD sequencer platform (Life Technologies, CA, USA). Sequence alignments were generated
197 by mapping SOLiD color space reads to *L. rhamnosus* GG genome (10) or *L. casei* BL23 (22) as
198 reference genomes, using the SOLiD BioScope software (Life Technologies, CA, USA) and the SAM
199 tools (36). In order to transfer annotation from a reference genome (GG or BL23) to an un-annotated
200 query genome, sequences were compared with 'nucmer' to identify regions that share synteny, those
201 regions were extracted out as base range in the query and base range in the reference genome. In-house
202 custom-made scripts were used to transfer annotation. The nucleotide sequence identity between
203 synteny blocks were more than or equal to 40%. In the case of the *L. rhamnosus* strains, initial
204 detection of Single Nucleotide Polymorphism (SNPs) and INsertion/DELetion (InDels) was performed
205 and chromosomal regions with identified mutations were further analyzed. We only considered
206 unequivocal SNPs with a sufficient sequence coverage (>18) and verified them by PCR amplification
207 using High-Fidelity Phusion DNA polymerase (Thermo Scientific, MA, USA) as per manual
208 instructions. The PCR amplicons were then sequenced and compared to the reference *L. rhamnosus*
209 strain GG. Orthologous genes between GG and BL23 genomes were calculated using blastp (37) with
210 the standard scoring matrix BLOSUM62 and an initial E-value cut-off of 1.10^{-4}. The score of every
211 blast hit was set into proportion to the best score possible, the score of a hit of the query gene against
212 itself. This resulted in a so-called score ratio value (SRV) between 0 and 100 that reflected the quality
213 of the hit much better than the raw blast bit score (38). Two genes were considered orthologous if it
214 existed a reciprocal best blast hit between these genes, and both hits had an SRV > 35. Genomes were

assigned to COGs using rps-blast (Reverse Position Specific blast) and NCBI's Conserved Domain Database (CDD).

Primer Extension. We used primer extension analysis to identify the transcriptional start site (TSS) and the promoter region of the SpaCBA pili gene cluster. We followed the same procedure as previously described by Tu *et al.* (39). Briefly, 5'-6-carboxyfluorescein (FAM)-labelled cDNA was generated from 2 µg total *L. rhamnosus* GG RNA using a FAM-6-labeled primer (5'-GTACCATTAGCATCGGTTTG-3') (Oligomer Oy, Finland) and RevertAid™ Premium Reverse Transcriptase Kit (Thermo Scientific, MA, USA) as per manufacturer's instructions. The cDNA mixture was then run on an ABI 3730 capillary sequencer in parallel with a Sanger sequencing reaction using the same primer.

233 **Results and Discussion**

234 **Isolation and Metabolic Characterization of the *Lactobacillus* Strains.** The *L. rhamnosus* strains
235 LrV and LrI were isolated from food and pharmaceutical products commercialized as carrying *L.*
236 *rhamnosus* GG under brand names Vifit (Friesland Campina, The Netherlands) and Idoform (Ferrosan,
237 Denmark), respectively. The *L. casei* strains LcY and LcA were isolated from the food drinks branded
238 as Yakult (Yakult Honsha Co., Japan) and Actimel (Danone, France), respectively. Genomic DNA was
239 isolated and used for 16S rRNA gene sequencing and *tuf* gene analysis (34) that confirmed the
240 correctness of their species identification (data not shown). Subsequently, the metabolic properties of
241 the isolated *Lactobacillus* strains were compared with those of the well-characterized *L. rhamnosus* GG
242 and *L. casei* BL23 (Fig. 1). Both isolated *L. rhamnosus* strains showed identical sugar utilization
243 profiles characteristic for that of *L. rhamnosus* GG, including the capacity to convert L-fucose but the
244 inability to use D-lactose or L-rhamnose (10). In contrast, the *L. casei* strains showed considerable
245 variation, indicating that the isolates from probiotic products are not identical. Specifically, they all
246 converted D-lactose, D-maltose and L-sorbose but not L-fucose while the strain LcY isolated from the
247 Yakult product could also remarkably utilize D-melibiose and sucrose (Fig. 1).

248 **Bile Salt Resistance, Mucus Binding and Intestinal Signalling of *Lactobacillus* strains**. Several
249 features that have been recognized as probiotic properties were analyzed for the *Lactobacillus* strains
250 isolated from probiotic products in comparison with the well-studied *L. rhamnosus* GG and *L. casei*
251 BL23. The bile salt resistance was tested in a plate agar system or media containing taurocholic and
252 glycocholic acids derived from the used Ox gall bile. All *L. casei* strains (BL23, LcY and LcA) and all
253 *L. rhamnosus* strains (GG, LrV and LrI) were found to be moderately resistant. This is in agreement
254 with previous data on the bile sensitivity of Lactobacilli (31, 40) and detailed information on the

255 proteomic bile response of the *L. casei* strains(32), including those used to produce Yakult and Actimel
256 (30) and *L. rhamnosus* GG (41).

257 The adhesion ability to human mucus of a variety of *Lactobacillus* strains marketed as probiotics has
258 previously been reported to be highly variable with *L. rhamnosus* GG as the highest binding strain
259 tested (42). Hence, we compared the adhesion properties of all used *Lactobacillus* strains to human
260 mucus (Fig. 2). Indeed, all *L. rhamnosus* isolates (GG, LrV and LrY) showed very high mucus binding
261 properties, while the *L. casei* strains showed only moderate (BL23) or virtually no binding (LcY and
262 LcA).

263 Finally, we compared the capacity of the strains to signal *via* Toll-like Receptors (TLRs) in a
264 mammalian cell line. No significant signalling response was found *via* the TLR4 and TLR5 receptors,
265 which is line with the absence of their key ligands (the lipopolysaccharides and the flagellins,
266 respectively) in these lactobacilli strains (data not shown). In contrast, specific and reproducible
267 responses were obtained in a TLR2 reporter cell line where the NF-κB-response was determined *via* a
268 reporter fusion (Fig. 3). All *L. rhamnosus* strains (GG, LrV and LrI) showed significant and similar
269 signalling *via* TLR2, *L. casei* BL23 showed moderate response but the isolates LcY and LcA, showed
270 only background signalling in this *in vitro* system.

271 This different signalling response *via* the TLR2 receptor is remarkable since the cell wall components
272 such as peptidoglycan and lipoteichoic acids that signal to TLR2 are generally present in all
273 Lactobacilli. One possible explanation could be that the non-signalling strains LcY and LcA, lack the
274 pili that are known to be present in *L. rhamnosus* GG (10) and that have recently found to be the
275 primary factors involved in promoting intestinal signalling (43). *L. rhamnosus* GG pili are decorated by
276 the mucus-binding protein SpaC (10, 17), and *L. rhamnosus* strain lacking the expression of pili do not

277 display any mucus binding ability, which may also explain the observed absence of adhesion to mucus
278 by the studied *L.casei* strains. Hence, we performed a comparative analysis of the genomes of the *L.*
279 *rhamnosus* isolates (GG, LrV, LrI) and the *L. casei* strains (BL23, LcA, LcY). However, this showed
280 clearly that the genomes of the *L. rhamnosus* strains (GG, LrV and LrI) and all *L. casei* strains (BL23,
281 LcA and LcY) contained identical sequences for the *spaCBA-srtC* gene cluster and therefore we
282 focused on a detailed analysis of their expression of these pili genes.

283 **Analysis of Pilus Gene-Encoded Cell Wall-Associated Proteins.** The pili of *L. rhamnosus* GG can be
284 detected by using antibodies against the major pilus protein SpaA or the mucus-binding protein SpaC
285 (17). Western blotting analysis using polyclonal SpaA antibody (Fig. 4) showed that the cell envelope
286 fractions of all *L. rhamnosus* strains (GG, LrV and LrI) contained the protein multimers characteristic
287 of pili with different sizes (17). In contrast, when the same experiment was applied on the *L. casei*
288 strains (BL23, LcA and LcY) no such SpaA multimers or even monomers were detected (Fig. 4). This
289 was confirmed by overexposing the Western blots or by spotting whole cells, supernatants or cell-
290 extracts of the *L. casei* strains followed by incubation with anti-SpaA or anti-SpaC antibodies (data not
291 shown). Hence, we conclude that none of the *L. casei* strains is producing the mucus-binding pili
292 characteristic for the *L. rhamnosus* strains GG, LrV and LrI.

293 Subsequently, we used immunogold labelled anti-SpaA antibodies in an immuno-EM experiment
294 aimed to identify the ultrastructure of the pili (Supplementary Figure S1). As expected, all
295 *L .rhamnosus* strains produced similar pili phenotype characteristic of *L. rhamnosus* GG (17) while no
296 such pili structures could be identified in any of the *L.casei* strains. Altogether, these experiments
297 indicate that while *L. rhamnosus* GG and its re-isolates from probiotic products produce the mucus-
298 binding pili, these are not present in *L. casei* BL23 or strains LcA and LcY, isolated from the probiotic

products Actimel and Yakult. In addition, the use of SpaC anti-serum in mucus-binding assays abolished the mucus-binding ability of LrV and LrI, as previously reported in GG (10). This further supports the important role of the SpaCBA pili in the interaction with human intestinal mucosa.

Comparative Genome Analysis of *L. rhamnosus* and *L. casei* strains. To further characterize the *Lactobacillus* strains isolated from probiotic products, we determined their genome sequences and analysed these based on a comparison of the well-established genomes of *L. rhamnosus* GG and *L. casei* BL23 (10, 22). The 3 Mb genomes of the latter strains are similar sized (10, 22), among the largest in the *Lactobacillus* genus (4) and include no plasmids unlike *L. rhamnosus* LC705 (10) and *L. casei* ATCC 334 (21). Moreover, the genomes of *L. rhamnosus* GG and *L. casei* BL23 show a high degree of synteny, with only few regions disrupted throughout the chromosome (as revealed by ACT comparisons and Gepart dot-plot alignments; Fig. 5). These regions mostly consist of genomic islands encoding sugar transport system and prophages. Protein predictions indicated that at total of 2180 proteins with a high amino acid identity score were shared (including the identical *spaCBA-srtC* gene cluster), while 836 or 835 proteins were strain-specific for *L. rhamnosus* GG and *L. casei* BL23, respectively (Fig. 6). The COG distribu tion revealed that a significant part of strain-specific genes were involved in carbohydrate transport and metabolism, supporting the observed metabolic differences (Figs. 5 and 6).

Subsequently, the genomes of the *L. rhamnosus* strains (LrV and LrI) and the *L. casei* strains (LcY and LcA) were analyzed by SOLiD sequencing with paired-ends and single reads (50bp forward reads and 35 bp reverse reads) totalling 8.9-12 million reads and amounting to over 100 Mbp for each genome. The SOLiD sequencing reads were mapped to the *L. rhamnosus* GG and *L. casei* BL23 genomes, providing sufficient information to gain insights in gene content, genetic order and single nucleotide

polymorphisms. In SOLiD mapping approach, tandem repeats, mononucleotide repeats and low complexity sequences present in the genomes may not be correctly mapped in some cases, as previously reported (44, 45). Using the annotation method described above, comparative analysis of the genomes of the *L. rhamnosus* isolates and that of *L. rhamnosus* GG (10) revealed strains LrV and LrI to be virtually syntenous at the genomic level. We also identified 4 and 2 SNPs to be present in LrV and LrI, respectively (Table 2). Remarkably, the 2 SNPs were identical and located in intercistronic regions, suggesting that these are either hot spots for mutation or that the strain isolated from the Vifit product has been derived from that recovered from the Idoform product and later acquired two additional SNPs. These additional 2 SNPs are not expected to have an impact on the phenotype as observed in the present study. These were either located in a lipoprotein gene or affected the *glvA* gene that is involved in the dysfunctional maltose metabolism (Fig. 1). This illustrates the genomic stability of *L. rhamnosus* GG used in food products, including Idoform and Vifit. It is noteworthy that in the course of this analysis we identified two sequencing errors present in the original *L. rhamnosus* GG genome sequence (10), located at the coordinates 615,483 bp (T>C) and 1,883,242 bp (C>A). The deposited NCBI GenBank sequence (accession number FM179322) was corrected accordingly.

As no genomic information relating to the *L. casei* strains used in Actimel and Yakult was available, all SOLiD reads of the LcA and LcY strains were mapped onto the genome of *L.casei* strain BL23 (22). This showed that *L. casei* BL23 and strain LcA isolated from Actimel are highly similar and all genes of strain BL23 were found to be present in *L. casei* LcA, including the identical *spaCBA-srtC* gene cluster. A closer look at the consensus sequence shows that there were only 158 undetermined nucleotides, suggesting potential SNPs or InDels. These were not further addressed in this study as they need more extensive high resolution sequence analysis. In contrast, a total of 34 genes from *L. casei* BL23 were not shared with strain LcY isolated from Yakult, indicating a further phylogenetic distance

344 than strain LcA isolated from the Actimel product, which is reflected at a functional level when
345 comparing the metabolic capacity of the *L. casei* strains (Fig. 1). The genes lacking in strain *L. casei*
346 LcY include 34 genes encoding a prophage (Table S1). However, the *spaCBA-srtC* gene cluster was
347 intactly present in the genome of *L. casei* LcY. In addition, the large number of approxim ately 70
348 undefined nucleotides suggested more SNPs and InDels that differentiated the *L. casei* BL23 and LcY
349 strains. With the genomic resequencing approaches used here we could not identify genes not present
350 in BL23 but a more comprehensive high resolution sequence analysis of all four strains that is presently
351 ongoing indicated that we were able to cover 99 and 97 % of the genomic information present on the *L.*
352 *casei* LcA and LcY isolated from the Actimel and Yakult strains, respectively (unpublished data).

353 **Identification of the transcriptional start site of the *spaCBA* pili operon.** As all of the tested *L.*
354 *rhamnosus* but none of the *L. casei* strains were producing the pili, in spite of the high conservation and
355 sequence identity of the *spaCBA-srtC* pili gene cluster (Fig. 7), we inspected the sequence upstream of
356 this gene cluster (Fig. 7). A number of differences were evident that may affect the expression of the
357 pili genes, notably those in the presumed promoter region. To define the transcription initiation of this
358 cluster, we performed primer extension analysis, resulting in identifying the promoter region of the
359 *spaCBA* pili gene cluster in *L. rhamnosus* GG. We observed that the transcriptional start site is located
360 47 nucleotides upstream the *spaC* start codon (ATG). A putative -10 and -35 region was proposed (Fig.
361 7). Interestingly, the putative promoter region identified in *L. rhamnosus* GG significantly differs from
362 the sequences present in the *L. casei* strains: the differences result in loss of the consensus -35 and -10
363 regions, and the transcriptional start site (TSS). In spite of the fact that the *L. rhamnosus* promoter
364 sequence does not resemble the canonical promoter, it shows high expression of the different pili genes.
365 The possibility that alternative sigma factors are used by this promoter is unlikely as these have not
366 been identified as major control system in LAB (46). Moreover, the spacing between the predicted

Shine-Dalgarno sequence and the initiation codon of the *spaC* gene is 2 nucleotide longer in the *L. casei* genomes, suggesting that apart from a transcriptional defect also the translation would be less efficient than in *L. rhamnosus* strains. This all would explain the absence of any detectable pili in the *L. casei* strains and correlate with the absence of mucus binding. In *L. rhamnosus* GG and the virtually identical strains LrV and LrI, an IS element is present upstream the *spaC* gene in contrast with *L. casei* strains (26), suggesting that the integration of the IS element resulted in the activation of the pili gene expression in these strains but not in the *L. casei* strains. Such transcriptional activation is reminiscent of various other bacterial systems, where gene expression is enhanced or altered by the introduction of IS elements (47, 48).

Conclusions. We characterized four probiotic-marketed strains at a genomic and phenotypic level. The two *L. rhamnosus* strains LrV and LrI were virtually similar to GG in terms of genomes and phenotypes, showing the product stability of the widely used probiotic strain *L. rhamnosus* GG. Remarkably, the identification of SNPs also suggested the *L. rhamnosus* strain isolated from the Vifit probiotic product may have been derived from the strain recovered from the Idoform product or indicates the presence of hot spots for mutations. The two *L. casei* strains isolated showed more heterogeneity compared to *L. casei* BL23 regarding genome content and carbohydrate utilization. Interestingly, when looking at the presence of SpaCBA pili structures in *L. rhamnosus* and *L. casei* strains by immunoblotting analysis, electron microscopy and mucus binding assay, only *L. rhamnosus* strains were displaying functional pili that could correlate to their mucus binding abilities and possibly responsible to the TLR-2 response. The identification of the transcriptional start site of the *spaCBA* operon also suggested that the expression of pili was triggered by the insertion of the IS element in *L. rhamnosus* strains, in contrast with *L. casei* strains. This single horizontal gene transfer, *i.e.* insertion of the IS element upstream *spaC* gene, appeared to have a significant impact on the evolution of *L.*

rhamnosus species by conferring a beneficial trait to colonize and persist mucosal-associated niches, such as the human gastro-intestinal tract.

Acknowledgements

The present study was financially supported by the ERC grant Microbes Inside, the Center of Excellence in Microbial Food Safety Research (CoE-MiFoSa), Academy of Finland (grant 141140) and the University of Helsinki. François P. Douillard was sponsored by a postdoctoral research fellowship from the Academy of Finland (grant 252123). We are also grateful to Pia Rasinkangas, Elina Nummenmaa, Päivi Laamanen, Kirsi Lipponen and Eeva-Marja Turkki for excellent technical assistance and Edward Alatalo for help in bioinformatics. We thank Dr. Vincente Monedero from the Institute of Agrochemistry and Food Technology (Valencia, Spain) for kindly providing the *L. casei* strain BL23. Dr. Satu Vesterlund, University of Turku, and MD Heikki Huhtinen, Turku University Central Hospital, Turku, Finland, are thanked for collecting the human intestinal mucus samples.

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580 **Figure Legends**

581 **Figure 1.** Metabolic profiles of the studied *Lactobacillus* strains. The fermentative capabilities of each
582 strain are color-coded respectively in black and grey for complete and partial carbohydrate utilization.
583 Carbohydrates that were not fermented are shown in white. The results are those observed after 48 h
584 incubation. Not used by any of the strains were Glycerol, Erithritol, D-Xylose, L-Xylose, Methyl-ß-D-
585 Xylopyranoside, L-Rhamnose, Methyl-αD-Glucopyranoside, Inulin, D-Raffinose, Amidon, Glycogen,
586 Xylitol, D-Fucose, D-Arabitol, L-Arabitol, 2-Ketogluconate Potassium, 5-Ketogluconate Potassium.

587 **Figure 2.** Binding profiles of *L. rhamnosus* and *L. casei* strains expressed as percentage (%) adhesion
588 to human intestinal mucus. The binding data are expressed as means ± standard deviation. The
589 differences between data sets are considered significant ($p \leq 0.0001$).

590 **Figure 3.** Response of HEK-Blue™ hTLR2 cells to *L. rhamnosus* and *L. casei* strains. HEK-Blue™
591 hTLR2 cells were stimulated with one of the six LAB strains for 24h, after which cell culture
592 supernatant was incubated for 1h, 2h and 3h for detection NF-κB activation. NF-κB-induced SEAP
593 activity was measured by spectrophotometer and converted in fold-changes. The data are expressed as
594 means ± standard deviation. Legend: PAM for PAM(3)CSK(4).

595 **Figure 4.** Immunoblotting analysis of cell-wall associated proteins of respectively *L. rhamnosus* GG
596 (lane 1), LrV (lane 2), LrI (lane 3), *L. casei* BL23 (lane 4), LcY (lane 5) and LcA (lane 6). The
597 membrane was probed with polyclonal serum directed against the SpaA pilin subunit. HMWL stands
598 for High Molecular Weight Ladder.

599 **Figure 5.** Genomic comparison of *L. casei* BL23 and *L. rhamnosus* GG. Panel (A): ACT (Artemis
600 Comparison Tool) comparison of *L. rhamnosus* GG (bottom chromosome) and *L. casei* BL23 (top

chromosome) (49). Red and blue bars respectively indicate similar regions between GG and BL23 (BlastN hits) that have the same orientation or have been inverted. Panel (B): Dot plot alignments of GG and BL23 using Gepard (50).

Figure 6. Comparative genomic overview of *L. rhamnosus* GG and *L. casei* BL23. Panel (A) shows the number of shared and strain-specific genes. Panel (B) shows the COG distribution of the different subset of genes shown in Panel (A).

Figure 7. SpaCBA pili cluster comparison in *L. rhamnosus* and *L. casei* strains. Panel (A) Blast results and corresponding amino-acid conservation percentage are indicated for each gene. The presence of different motifs is color/pattern-coded: green arrow for sortase, white arrow for pili subunit, blue for secretion signal, yellow for LPxTG motif, purple for von Willebrand type A domain and red for Cna protein B-type domain. Panel (B) Primer extension analysis of the SpaCBA pili promoter. Is shown the sequencing chromatogram and the peaks (yellow) detected during the analysis. Panel (C) shows the sequence alignment of the upstream region of the *spaC* gene in *L. casei* and *L. rhamnosus* strains with the position of the transcriptional start site, the putative -10 and -35 regions and also the ribosome binding site (RBS). Nucleotides highlighted in red in *L. casei* BL23 sequence differ from GG.

621 ## Tables

622 **Table 1.** Strains used in this study.

Strain name	Functional product	Product category	Origin/Manufacturer
L. rhamnosus GG (ATCC 53103)	Gefilus product family	buttermilks, yoghurts, milk, fruit drinks, dairy drinks and fermented whey-based drinks	Valio Ltd culture collection (FI)
L. rhamnosus LrV	Vifit product family	yoghurts and drinkable yoghurts	Friesland Campina (NL)
L. rhamnosus LrI	Idoform	tablets	Ferrosan (DK)
L. casei BL23 (ATCC393)	n/a	dairy product	(51)
L. casei LcY	Yakult	fermented milk drink	Yakult Honsa Co. (JP)
L. casei LcA	Actimel	fermented milk	Danone (FR)

623 n/a: not available

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625

626

627 **Table 2.** Summary of SNPs identified in *L. rhamnosus* LrV and LrI strains. A cross indicates in which strain the
628 mutation occurred. n/a: not applicable

SNP coordinate in GG	LrI	LrV	Nucleotide change	Gene	AA change	Description
1,030,390	x	x	T>G	LGG_01017	H294Q	Lipoprotein
1,373,568		x	G>A	n/a	n/a	Intercistronic region between converging LGG_1372 (conserved protein) and LGG_1371 (conserved protein)
2,649,651		x	G>T	n/a	n/a	Intercistroninc region upstream region of LGG_01853, ABC transporter, substrate-binding protein
2,765,383	x	x	G>A	LGG_02701	H98N	Maltose-6'-phosphate glucosidase *glvA*

629

Fig.1

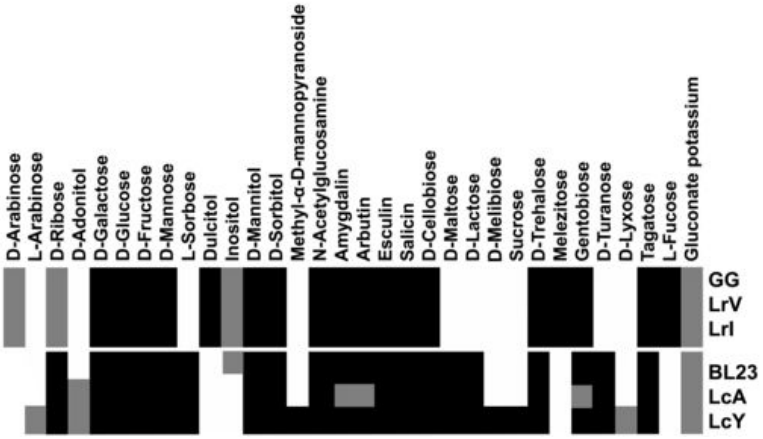


Fig.2

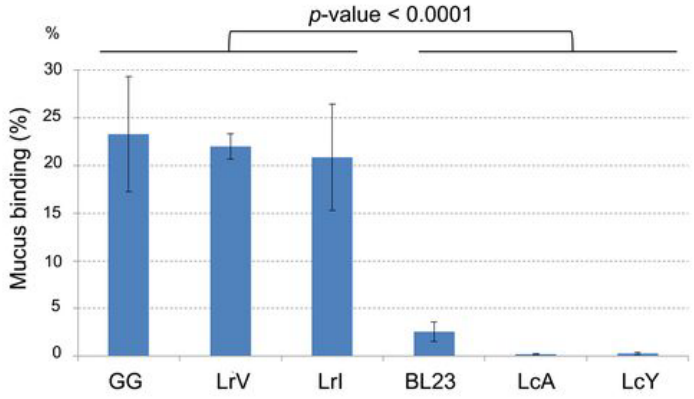


Fig.3

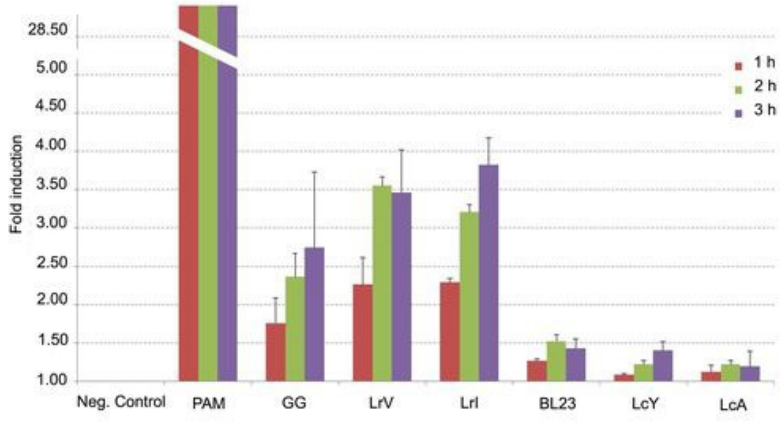


Fig.4

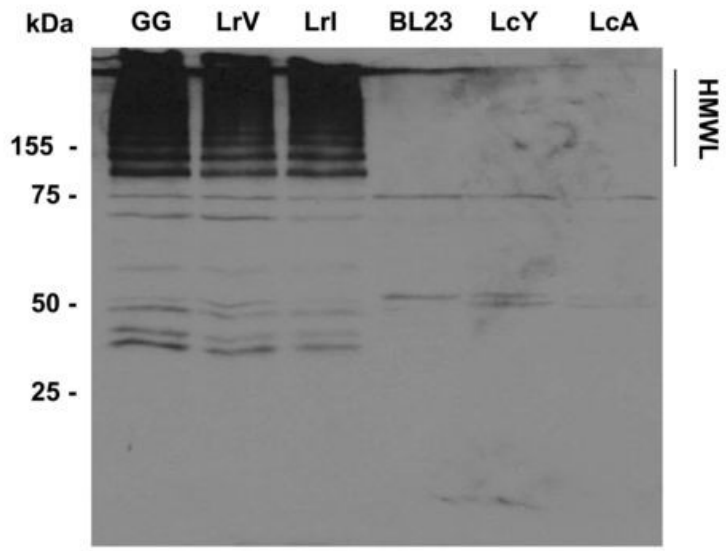

kDa
GG
LrV
LrI
BL23
LcY
LcA
155 -
75 -
50 -
25 -
HMWL

Fig.5

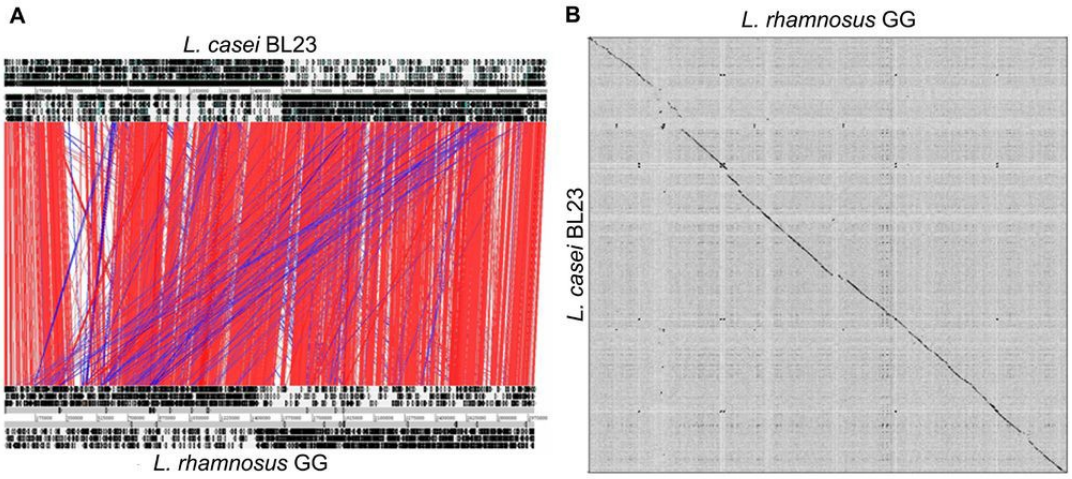

A
L. casei BL23
L. rhamnosus GG
B
L. rhamnosus GG
L. casei BL23

Fig.6

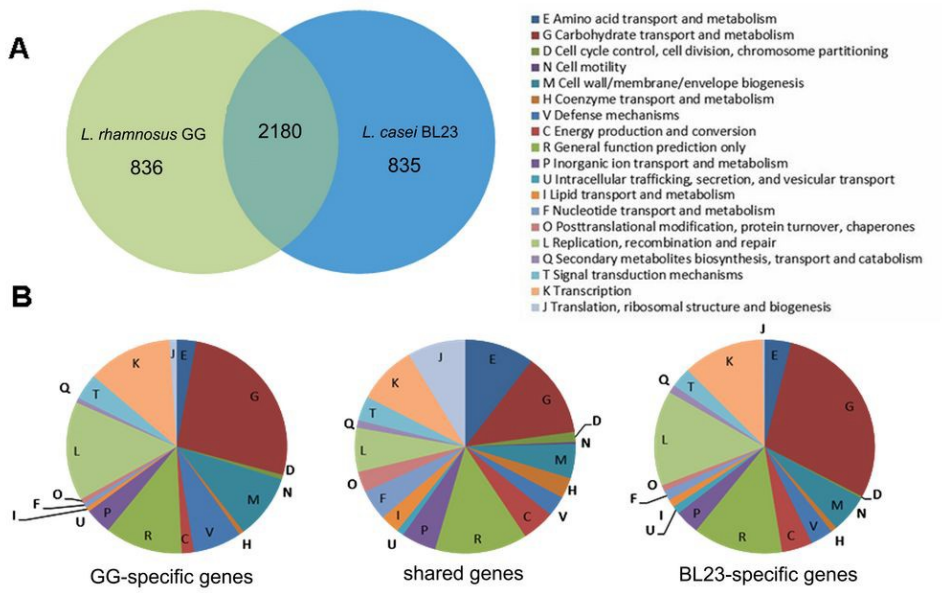
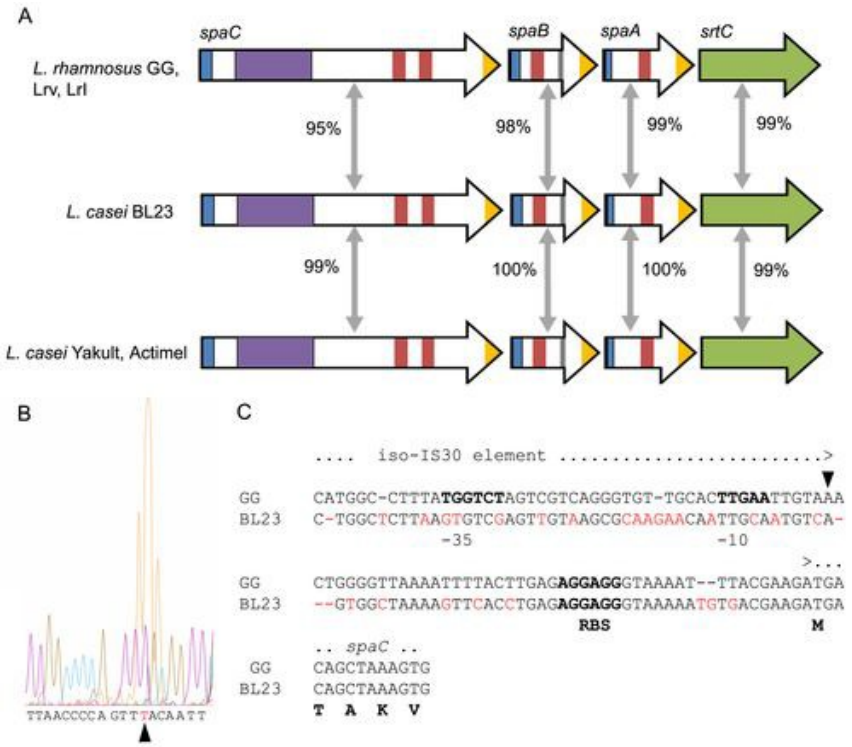
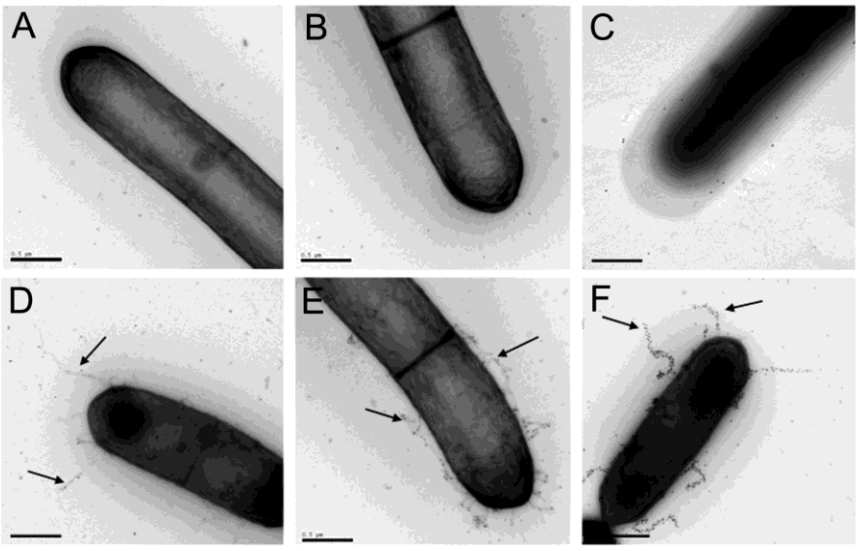

A
L. rhamnosus GG
836
2180
L. casei BL23
835
E Amino acid transport and metabolism
G Carbohydrate transport and metabolism
D Cell cycle control, cell division, chromosome partitioning
N Cell motility
M Cell wall/membrane/envelope biogenesis
H Coenzyme transport and metabolism
V Defense mechanisms
C Energy production and conversion
R General function prediction only
P Inorganic ion transport and metabolism
U Intracellular trafficking, secretion, and vesicular transport
I Lipid transport and metabolism
F Nucleotide transport and metabolism
O Posttranslational modification, protein turnover, chaperones
L Replication, recombination and repair
Q Secondary metabolites biosynthesis, transport and catabolism
T Signal transduction mechanisms
K Transcription
J Translation, ribosomal structure and biogenesis
B
GG-specific genes
shared genes
BL23-specific genes

Fig.7


A
spaC
spaB
spaA
srtC
L. rhamnosus GG, Lrv, Lrl
95%
98%
99%
99%
L. casei BL23
99%
100%
100%
99%
L. casei Yakult, Actimel
B
TTAACCCCA GTTTACAATT
C
.... iso-IS30 element .....>
GG CATGGC-CTTTATGGTCTAGTCGTCAGGGTGT-TGCACTTGAATTGTAAA
BL23 C-TGGCTCTTAAGTGTCGAGTTGTAAGCGCAAGAACAATTGCAATGTCA-
-35 -10
>...
GG CTGGGGTTAAAATTTTACTTGAGAGGAGGGTAAAAT--TTACGAAGATGA
BL23 --GTGGCTAAAAGTTCACCTGAGAGGAGGGTAAAAATGTGACGAAGATGA
RBS M
.. spaC ..
GG CAGCTAAAGTG
BL23 CAGCTAAAGTG
T A K V


A
B
C
D
E
F

1

2

3

4

Comparative genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains from human and food origin

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Abstract

Background

Known for its use in products marketed as probiotics, *Lactobacillus rhamnosus* is a lactic acid bacterium that is found in a large variety of ecological habitats, *e.g.* the oral cavity, the human gastro-intestinal tract, and various food products, including artisanal cheeses. To gain insights into the genetic complexity and ecological versatility of the species *L. rhamnosus*, we examined the genomes and phenotypes of 100 *L. rhamnosus* strains that were isolated from diverse sources.

Results

The genomes of 100 *L. rhamnosus* strains were analyzed and compared based on SOLiD sequence analysis of their 3 Mb genomes. These strains were phenotypically characterized for a wide range of metabolic, antagonistic, signalling and functional properties. Phylogenomic analysis showed multiple sublineages of the species that could partly be associated with their ecological niches. We identified seventeen highly variable regions, with a total size of approximately 200 kb, in the *L. rhamnosus* genome that encode functions related to lifestyle, *i.e.* carbohydrate transport and metabolism, production of mucus-binding pili, bile salt resistance, prophages and CRISPR adaptive immunity. Integration of the phenotypic and genomic data also revealed that some *L. rhamnosus* strains possibly resided and evolved in multiple niches, illustrating the dynamics of bacterial habitats.

Conclusions

The present study showed a duality in the evolution of *L. rhamnosus* between human- (mucosal surfaces) and food-associated niches. The human strains were genetically

different from those strains marketed as probiotics or encountered in foods and, showed a remarkable versatility to persist in a variable environment in terms of nutrients, bacterial population and host. The food-associated strains were adapted to stable nutrient-rich niches, showing loss of non-essential biological functions that would confer antimicrobial resistance, adaptability and fitness to a broad range of habitats.

Keywords

L. rhamnosus, genetic diversity, ecology, niche adaptation

Background

The current development and application of high-throughput sequencing technologies allow to intensively investigate complex microbial ecosystems, such as the human gastro-intestinal (GI) microbiota, consisting of over 3 million genes from mainly Gram-positive bacteria [1-4]. This and other metagenomic approaches obviate the necessity to culture bacterial isolates to comprehend the richness and the diversity of such ecosystem. However, detailed analysis at the strain level still requires isolation and growth of bacterial residents. Gram-positive lactobacilli are naturally found among ~1000 phylotypes identified in the human intestinal tract [2], but only a fraction is represented in the present metagenomic sequences that derive from faecal samples. Lactobacilli mainly reside in the intestinal mucosa and were detected in the ileum metagenome [5]. As a consequence of their health-promoting properties in the human intestinal tract, lactobacilli are increasingly used in food production, food preservation and nutritional complement formulation [6-10]. One of the most used and documented lactobacilli marketed as a probiotic is *Lactobacillus rhamnosus* GG (LGG), that has been isolated from the human intestine and characterized at the genome level [11-13]. LGG possesses remarkable abilities to colonize and persist in the human intestinal mucosa, as it produces pili that are decorated with the mucus-binding protein SpaC [14-16]. This significantly impacts the intestinal microbiota, *via* the displacement of pathogenic bacteria [17], modulation of epithelial barrier functions [18] and potential stimulation of the host immune system *via* bacteria-host surface molecule crosstalk [8, 18-20]. Since the host-probiotic bacteria interaction has a pivotal role in the resulting health-promoting effects for the host, much research effort now focuses on the characterization of the different interaction players, as well as metabolic properties and host-signalling components of *L. rhamnosus* [20].

However, no studies have actually addressed the diversity of the species *L. rhamnosus*, in spite of its extensive use in a variety of food products. While some *Lactobacillus* species have been found in only one dedicated niche, such as the milk-adapted *L. helveticus* [21], other lactobacilli such as *L. rhamnosus*, *L. casei* or *L. plantarum* have the capacity to colonize multiple habitats [7, 22-24]. More specifically, *L. rhamnosus* has been isolated from a large variety of ecological niches, *e.g.* human intestinal tract, blood, vagina, oral cavity and cheese, exemplifying its remarkable ecological adaptability [11, 25-28].

Genome sequencings of a number of lactobacilli revealed that the adaptation of lactobacilli to diverse ecological niches is promoted by the acquisition of new genes/functions by horizontal gene transfers and the decay or loss of non-essential genes/functions [22, 24, 29, 30]. The domestication of dairy lactobacilli species is a typical example of a niche specialization, where milk-adapted strains have unusually high number of pseudogenes, reflected by the loss of metabolic pathways and transport systems non-essential in dairy niches rich in nutrients [29, 31]. In contrast, organisms from the intestinal tract, a very dynamic habitat in terms of nutrient availability and bacterial population, have broader metabolic capacities and lifestyle traits essential for survival, persistence and colonization in the gut, *e.g.* bile resistance [32, 33], anti-microbial activity [34], and mucus-binding pili expression [11]. In some cases, gene sets could even be specifically linked to a particular ecological niche, *i.e.* gut *vs.* dairy environment, as reported for the related *L. acidophilus* and *L.helveticus* [29].

The present study of the species *L. rhamnosus* aimed at: *(a)* investigating the genomic diversity and evolution of the species, *(b)* examining the lifestyle and metabolic diversity of *L. rhamnosus* in regards to various ecological niches, *(c)* identifying and

analysing variable chromosomal regions possibly associated to phenotypic and/or lifestyle traits. Four complete *L. rhamnosus* genomes have been fully sequenced and assembled, allowing us to have a glance at the diversity of the species [11, 35, 36]. In an effort to further comprehend the diversity and versatility of *L. rhamnosus* species, we compared the genomes and phenotypes of 100 *Lactobacillus rhamnosus* strains that were isolated from different ecological niches. This study represents the first large-scale genomic and functional analysis of the *L. rhamnosus* species, providing important findings on its genetics and also on its lifestyle and metabolic adaptability from an ecological and evolutionary perspective.

Results and discussion

General genomic features of the species *L. rhamnosus*

To comprehensively depict the phenotypic and genomic diversity of the *L. rhamnosus* species, 100 *L. rhamnosus* strains were isolated from a broad spectrum of ecological niches, *e.g.* 72 strains of various sites of the human body (oral cavity, vaginal cavity, blood and intestinal tract) and 28 strains of food origins, including artisanal cheeses and products marketed as probiotics (Additional Table S1). The genomes of all strains were characterized using the SOLiD sequencing technology and a total of over 800 million reads were mapped onto the LGG chromosome, allowing further comparative genomic analysis and data mining as described in the Methods section. The number of shared genes between LGG and the 100 *L. rhamnosus* isolates ranged from 2622/3016 (86.9%) to 3016/3016 (100%) genes with a median number of 2918/3016 (96.7%) genes (Figure 1). In terms of relative gene content, the food isolates showed a lower shared gene content with LGG, with a median number of 2807/3016 (93%), compared to human isolates, 2955/3016 (97.9%), indicating that most food isolates are phylogenetically more distant from LGG. It is noteworthy that 11 strains of human origin, 3 strains isolated from products marketed as probiotics and only 1 strain isolated from artisanal cheese shared 100% of LGG gene content. However, it has to be kept in mind that orthologous genes present in these isolates may present mutations, *i.e.* single nucleotide polymorphisms, insertion and deletions that were not addressed in this study. Therefore, the presence of a gene may not necessarily reflect its functionality, as observed within these 11 strains, which showed significant phenotypic variations, indicating that these strains were not *L. rhamnosus* GG, excluding *L. rhamnosus* GG re-isolates VIFIT and IDOF (see below). Also, strain-specific genes are likely to be present in these isolates, conferring additional

192 phenotypic traits not present in LGG. Based on comparative gene content, the
193 phylogenetic analysis of the *L. rhamnosus* species showed a separate sublineage of
194 the species in 4 distinct clusters, where most food strains belong to the same lineage
195 (Figure 1). *L. rhamnosus* strains used in probiotic-marketed yogurt cultures share
196 common ancestries with other isolates of human origins, which concords with the
197 hypothesis that its genomes still reflect its adaptation to its original habitat, *i.e.* the
198 human intestinal tract [11]. The lineage separation of most food isolates was found to
199 appear early in the phylogenetic tree compared to that of the other isolates, suggesting
200 a duality in the evolution of the species and separates the cluster 1 dominated by of
201 food isolates from that of the other clusters dominated by strains derived from human
202 origin, from which two (clusters 3 and 4) consists predominantly of *L. rhamnosus*
203 strains closely related but not identical (apart from the 2 re-isolates) to LGG (Figure
204 1).
205 Based on the 100 mapped genomes, we defined a set of all orthologous genes that are
206 shared by all *L. rhamnosus* strains. We observed that the shared gene set (core) of the
207 *L. rhamnosus* species consists of 2419 genes, which represents 80.2% of LGG
208 genome. The larger set of strains used, the smaller the core genome, as typically seen
209 in the core-genome of *Streptococcus agalactiae* and other bacterial species [37, 38].
210 However, its size remained stable above ~20 genomes (data not shown). The full
211 listing of the core genes can be deduced from the non-core LGG gene list found in the
212 additional material (Additional Table S2). The full comparative genomic results for
213 each strain are also available in Additional Table S3. The present study focused on
214 comparative genomic and phenotypic analysis. Therefore, we did not use the SOLiD
215 sequencing information relative to strain-specific genes not present in LGG. Further
216 deep and full coverage sequence analysis of a subset of *L. rhamnosus* strains of

217 interest is now on-going to propose the pan-genome of the species *L. rhamnosus*. The
218 distribution of Clusters of Orthologous Groups of proteins (COG) was determined for
219 LGG genome, the *L. rhamnosus* core-genome and the non-core gene set (Additional
220 Figure S1). Relative gene counts of each COG category decreased compared to the
221 COG distribution in LGG. Although no major differences in the relative COG
222 distribution between the different subsets were found, it is noteworthy that 88 LGG
223 genes (31%) out of 288 genes assigned to the COG 'Carbohydrate transport and
224 metabolism' are not in the core genome and are predicted to encode mostly
225 phosphotransferase system (PTS) and other sugar transport systems, essential for
226 persistence in the intestinal tract. These genes were located in highly variable regions
227 of LGG chromosome, reflecting the metabolic diversity of the species *L. rhamnosus*
228 (Figure 2). In Table 1, the 17 most variable chromosomal regions in LGG include all
229 LGG genomic islands (GIs), typically rich in transposases and other mobile genetic
230 elements. In *L. rhamnosus* GG, 5 major genomic islands (GIs) were identified,
231 corresponding to ~80 genes [11]. The presence of these GIs greatly varies among
232 strains of the species *L. rhamnosus*, as observed previously for the strains LC705 and
233 GG [11]. This is corroborated in the present analysis with 100 other strains,
234 suggesting the important contribution of horizontal gene transfer events to the
235 diversity of the species. The variable regions in LGG were associated with specific
236 biological functions, including carbohydrate transport and metabolism, bile resistance,
237 production of exopolysaccharides (EPS), prophages, production of mucus-binding
238 SpaCBA pili structures, phages and plasmid immunity (CRISPR system) (Table 1).
239 These regions may be defined as lifestyle islands, as they specifically contribute to the
240 persistence and colonization in habitats, by encoding proteins involved in the
241 interaction and signallings with the host, the optimal use of available nutrients, and by

conferring protection against autochthonous phages and mobile genetic elements. Other variable regions consisted mostly of transposases and conserved proteins with no clear function and were not further addressed (Additional Figure S2). Unless specified, the strains shown in the different figures in the study were classified using the phylogenetic tree (Figure 1).

Metabolic islands, carbohydrate transport and metabolism and niches

Genomic analysis of the sequenced strains revealed the loss of 88 genes encoding various carbohydrate PTS system and metabolism-associated proteins among the 100 strains compared to LGG. To study the impact of these genomic characteristics, the metabolic capability to utilize different carbon sources was investigated, Carbohydrate utilization profiling showed that most *L. rhamnosus* strains use a large range of simple and complex carbohydrates (Figure 2). However, some differences may reflect their genomic diversity and also at some extent how they evolved in different ecological niches, by the acquisition or the loss of metabolic-associated genes. The ability to utilize carbohydrates mostly relies on the presence of functional transporter machinery and intact metabolic pathways. The clustering of *L. rhamnosus* strains (Figure 2) revealed strong associations between genome diversity, carbohydrate metabolism and their origins. Typically, strains belonging to LGG sublineage utilize D-arabinose, dulcitol and L-fucose, whereas other strains lost these functions but possesses the ability to use L-sorbose, D-maltose, D-lactose, D-turanose, methyl-α-D-glucopyranoside, L-rhamnose and D-saccharose (Figure 2). Hence, we detail the differences in carbohydrate utilization within the *L. rhamnosus* species below.

LGG genome harbors a tagatose-6-phosphate pathway (*lacABCD)* and a lactose PTS (*lacFEG*) but the antiterminator *lacT* and the phospho-β-galactosidase encoding *lacG* genes are altered and non-functional, preventing LGG from metabolizing D-lactose [11]. Strains belonging to LGG sublineage also show a poor or no ability to use D-lactose, whereas other isolates, including the dairy ones utilize lactose, a disaccharide exclusively found in milk and milk-derived products. We propose that the *lacT* and *lacG* genes have been kept intact in these strains, as lactose utilization represents an important carbon source and provide a real benefit for *L. rhamnosus* strains residing in these dairy niches. The maltose locus was predicted to be non-functional in LGG due to the insertion of a conserved gene (LGG_00950) between genes encoding the maltose-specific *malEFGK* transporter and the hydrolase (LGG_00949) [11]. Similarly to LGG, we found that most *L. rhamnosus* strains unable to use maltose also contained a maltose locus disrupted by LGG_00950. In contrast, the majority of strains belonging to other sublineage contained an intact maltose locus and were able to utilize maltose (Figure 2), indicating that the insertional inactivation by LGG_00950 played a significant role in *L. rhamnosus* species evolution. The maltose locus clearly appears to be non-essential in LGG and related mucosal surface-associated strains (Figure 2), suggesting that this genetic event did not hamper their ability to persist and colonize their niche. Comparative genome sequencing of LGG also showed that the rhamnose locus is altered: a galactitol-specific *gatABCD* PTS and a DeoR transcriptional regulator are missing and also the *rhaB* gene is duplicated, possibly explaining the inability to use rhamnose compared to some other *L. rhamnosus* strains, *i.e.* LC705 [11]. Combination of the genomic and metabolic data indicates that strains of the LGG sublineage similarly contain a defective rhamnose locus, whereas other strains harbour intact genes required for the transport and

291 metabolism of rhamnose. The loss or decay of the rhamnose locus in LGG and closely
292 related strains indicates that these genes are non-essential to persist in niches, such as
293 the human intestinal tract. In contrast, fucosylated compounds such as mucin
294 glycolipids and glycoproteins are commonly found in the intestinal tract and play an
295 important role in the human gut ecology, as a carbon source for intestinal bacterial
296 species [20]. Close inspection of the L-fucose metabolism revealed that a large
297 number of food-associated strains are unable to use L-fucose due to the lack of one or
298 more genes required to transport and metabolize L-fucose: the *fucU* and *fucI*
299 isomerases, *fcsR* fucose operon repressor and α-L-fucosidase (LGG_002652). Most
300 strains closely related to LGG retained the capacity to use L-fucose, whereas other
301 strains lost this ability, most likely as L-fucose is not abundant as in other niches, *i.e.*
302 bovine milk. Dulcitol, a polyol also known as galactitol, is also used by LGG and its
303 related sublineage (Figure 2). In most strains unable to use dulcitol, the function loss
304 was associated with the lack of an intact *gatABC* PTS system. Other carbohydrates
305 such as turanose and sorbose were not metabolized by strains related to LGG (Figure
306 2). In *L. rhamnosus* LC705, an intact sorbose *sorABCDEFGR* locus is present,
307 explaining its ability to utilize sorbose, whereas LGG lacks such machinery [11]. *L.*
308 *rhamnosus* strains with similar capabilities may therefore possess an intact sorbose
309 locus. Remarkably, the phylogenetically most distant *L. rhamnosus* strains from LGG
310 present a similar metabolic profile as *L. rhamnosus* LC705, which is an industrial
311 dairy strain [11]. This suggests that food-related strains characterized in the present
312 study underwent similar niche adaptation as LC705 in terms of acquisition, decay or
313 loss of genes in the food environments.
314

Diversity of the Clustered Regularly Interspaced Short Palindromic Repeats-Cas system: a spacer oligotyping analysis

CRISPR (clustered regularly interspaced short palindromic repeats) loci are present in a large number of prokaryote genomes [39], playing an important role in controlling horizontal gene transfer. It has been well established that some bacteria acquired the CRISPR-Cas system as a protection/immunization system against plasmid conjugation and phage predation [40-43]. The CRISPR-Cas system usually consists of a leader sequence, an array of CRISPRs interspaced by spacers and a *cas* gene cluster encoding the Cas protein complex (Figure 3, Panel A) [44]. The role and mechanistic of the CRISPR-Cas system in bacterial species has been extensively reviewed and indicate that the spacer sequence can be considered as a signature of past exposure to exogenous DNA [45]. *L. rhamnosus* GG has a single Type II-A CRISPR-Cas locus, consisting of 4 *cas* genes and one CRISPR array containing 24 spacers [11]. To determine whether the CRISPR sequences could be used as an indicator of a specific niche, we determined their diversity and the presence of the *cas* genes using LGG as a reference. CRISPR genotyping had been previously developed for epidemiological purposes and strain differentiation for *Mycobacterium tuberculosis* [46], enterohemorrhagic *Escherichia coli* [47] and *Salmonella enterica* [48]. We were able to generate a CRISPR profile (spacer oligotyping) for each strain and it revealed a high degree of diversity among the various strains (Figure 3, Panel B). Remarkably, all strains from the same sublineage were sharing a comparable CRISPR spacer set, whereas the more phylogenetically distant *L. rhamnosus* strains were only harbouring few LGG spacers and a poor conservation of the *cas* genes. The overall CRISPR-Cas typing analysis showed that strains from the same sublineage mostly shared identical CRISPR-Cas loci. Interestingly, strains F1489 and H4692 did not have any of LGG

spacers but some of the *cas* genes remained present, whilst strain H0047 lacked the entire CRISPR-Cas locus. It has to be kept in mind that only sequences homologous to the CRISPR-Cas locus from strain LGG could be identified, allowing the possibility that additional spacers, *cas* genes or even additional CRISPR loci may be present. To determine the function of the CRISPR-Cas system in protecting *L. rhamnosus* from exogenous DNA, BLASTN searches on all 24 spacers were performed against virus and plasmid at GenBank. Out of 24 spacers, 11 spacer sequences showed substantial sequence identity with plasmid or phage sequences (Additional Table S4). Eight spacer sequences fully or partially matched known bacteriophages genomes: *L. rhamnosus* phage Lc-Nu, *L. casei* phage φ AT3, *L. casei* phage Lrm1, *L. casei* phage A2 and *L. casei* phage PL-1. The identified CRISPR spacers thus belonged to phages from *L. rhamnosus* strains or closely related bacterial species, *i.e. L. casei*, highlighting the role of the CRISPR-Cas system as an immunity system against phage predation. Some spacers (4, 12, 21 and 22) have multiple phage hits, showing that the corresponding phage genomes share the same region, preventing us to predict from which bacteriophage these particular spacers were acquired. One match for plasmids was also found: the conjugative plasmid pSB102. The data also indicates that the CRISPR-Cas system may play a role in the *L. rhamnosus* species diversity by controlling horizontal gene transfer and, and providing phage resistance, thereby contributing to diversification of the species. Our data also showed that the degree of CRISPR diversity correlated with the phylogenetic mapping of isolates and at some extent with their ecological niche (Figure 3). Most food isolates shared only 6-7 spacers with LGG, indicating that the variety and the exposure to phages and other mobile genetic elements varies in each habitat, *i.e.* the intestinal tract and cheese. We anticipate that some of the food strains

may have an entirely different set of CRISPR sequences, representative of their own habitat and possibly additional CRISPR-Cas Types, as seen across the lactic acid bacteria [49].

Bile resistance, a persistence trait

All 100 *L. rhamnosus* isolates were tested for the resistance to bile salts, a property that is usually associated with the intestinal tract environment (Figure 4). When combining the bile salt resistance data with the phylogenetic tree, there was no clear association between species evolution and bile resistance (data not shown). A majority of *L. rhamnosus* strains were bile resistant (45% resistant and 30% moderately resistant). However, different bile resistance profiles were observed in each niche (Figure 4). A similar distribution was observed in strains isolated from blood, clinical samples and cheese, even though a slightly higher proportion of bile salt-sensitive strains could be observed in the food isolate group. As expected, all strains from the human intestinal tract were showing resistance to the bile salts, illustrating that such trait is essential for persisting in the intestinal tract. The vaginal isolates also showed similar traits, *i.e.* frequent bile resistance, suggesting that *L. rhamnosus* strains of the colonic microbiota may have colonized the vaginal cavity as previously reported [50]. The low number of isolates from oral cavities ($n = 3$) did not allow us to draw any conclusions, but revealed a different profile in terms of bile sensitivity. Similar bile resistance profiles were also observed in another set of isolates that belong to our *L. rhamnosus* collection (data not shown). One of the hyper-variable regions in LGG had genes encoding the taurine transport system *tauABC*, potentially involved in the bile salt conjugation. Seven out of 24 bile-sensitive strains had a defective *tauABC* locus, suggesting that the *tauABC* locus may

affect the bile sensitivity of these strains but most likely additional genes might be involved as well and still need to be identified.

Pilosotype and mucosal surface-associated niches

Pili in *L. rhamnosus* strains play a significant role in terms of interaction, colonization, persistence and potential signalling in the human intestinal tract [11-13]. The SpaCBA pili gene cluster is flanked by numerous IS elements, suggesting that *L. rhamnosus* might have acquired the SpaCBA pili gene cluster by horizontal gene transfer [30], where the integration of the *iso-IS*30 element had constituted a promoter that allowed the expression of the pili genes (submitted manuscript). It also indicates that this IS element-rich chromosomal region may be subject to important genetic recombination events within the species [11]. Hence, we examined the pili diversity among all isolates, providing a detailed picture on the conservation of the pili genes in each strain, since as little as one mutation is potentially sufficient to prevent the pili production or to affect the mucus binding abilities (Figure 5). Moreover, to support the genomic data, we investigated the mucus adhesion abilities of all *L. rhamnosus* isolates and also verified the presence of pili in a number of these strains by immunoblotting analyses ($n = 64$), electronic microscopy ($n = 10$) and *in vitro* inhibitory mucus binding assays ($n = 22$) (Figure 5, Additional Figures S3 and S4). The mucus binding capacity ranged from 0.05% to 29.9% in all tested strains and was clearly correlated with the presence of a functional SpaCBA pili gene cluster, as shown at both genomic and phenotypic levels. To further demonstrate that the mucus binding capacity of these strains was mediated by SpaCBA pili, we performed *in vitro* inhibitory binding assays on 22 SpaCBA-positive isolates using SpaC anti-serum (Figure 5 and Additional Figure S3) as previously described [11]. In all 22 strains

tested, including LGG, the presence of SpaC anti-serum significantly reduced mucus binding, suggesting that the pili are the major player involved in the interaction between *L. rhamnosus* and the host mucosa. Remarkably, some strains displayed significant mucus binding capacity but lacked the canonical SpaCBA pili structures, suggesting that other interaction players might be involved. Further characterization, including high resolution sequencings are needed to identify the proteins or structures that are involved in the interaction with the host. The food strain F0962 contained an identical SpaCBA pili cluster as LGG but showed the highest mucus binding of all *L. rhamnosus* strains examined in the study, suggesting that additional interaction components are also involved. The genes for the SpaCBA pili of the strains LGG, H1242, H6110 and F0962 are highly conserved but, however, with some subtle sequence differences. We propose that the sequence polymorphism of the pili genes in these strains might enhance mucus binding capacity or affinity. Alternatively, we cannot rule out that additional strain-specific traits might be involved in the mucus binding, especially in strain F1178 where the residual binding in the presence of SpaC anti-serum still remained high (Additional Figure S3). In contrast, those strains with poor mucus binding abilities appeared to have some remnants of pili genes more or less decayed (Figure 3). In strains H1275 and H4689, the SpaCBA pili gene cluster is highly conserved (~98-99%), but show a very poor binding, indicating that the pili production may be impaired by critical mutation(s).

The *L. rhamnosus* strains were further classified according to two main criteria, *i.e.* their ecological niche and their pilosotype, defined as the presence of pili genes that encode functional pili. Pilosotype of all isolates was determined using both genomic and phenotypic data (Table 2). The results, indicate that the production of a functional SpaCBA pili was significantly more prevalent in human isolates (40% or 29/72) than

in food isolates (18% or 5/28), suggesting that the expression of pili is not an essential traits for food-associated strains. The SpaCBA pilosotype was even less prominent (only13%) when the isolates from products marketed as probiotics were omitted. The loss of the pili gene cluster in food strains reflects a niche specialization of these strains to a habitat where pili structures are not essential and do not bring any benefit for persistence and colonization. In contrast, the human strains, mostly the ones isolated from the human intestinal tract, produce SpaCBA pili, which would confer the ability to efficiently colonize and persist in the intestinal tract. While the presence of pili is prevalent in intestinal isolates, it is, interestingly, not the case for all intestinal isolates. None of the strains originated from the oral cavity and the vagina possesses functional pili, indicating that such trait may not be required in these two ecological niches. Our observations support the hypothesis that the human-mucus binding properties of pili may be an advantage to the bacterial cells to persist in the intestinal niche, in particular the intestinal tract, but may be lost in strains evolving in other ecological niches, such as milk-based products, through the decay or loss of the non-essential SpaCBA pili gene cluster.

Cross-talk between *L. rhamnosus* and intestinal cells

Due to the intimate interaction between *L. rhamnosus* and the intestinal mucosa [20], we studied the potential signalling pathways that could be triggered by the *L. rhamnosus* strains. This was realized by determining the signal transduction in intestinal epithelial cells *via* Toll-like Receptors (TLRs) TLR-2, TLR-4 and TLR-5. All 100 isolates were tested for signallings *via* TLR-4 and TLR-5 receptors, but no significant responses were observed, which is in agreement with the identified ligands for these two TLRs, *i.e.* lipopolysaccharides and flagellins (data not shown). Clearly,

466 *L. rhamnosus*-host signallings are mediated through different receptors. Signalling *via*
467 the TLR-2 receptor in *L. rhamnosus* species was observed and greatly varied among
468 isolates (Additional Figure S5). More than half of the isolates mediated a TLR-2
469 response very similar to the level observed for strain LGG after 1h (fold-induction of
470 ~1.5). Six strains (H6111, H0009, H4692, H1311, H1226 and H1131) gave a stronger
471 signal in this assay system. We did not determine the nature of the ligand recognized
472 by TLR2 but assume in analogy with what has been found in LGG that the signalling
473 is mediated by the lipoteichoic acids [51]. The levels of TLR2 signalling could not be
474 correlated with any other traits, such as EPS production, pili production or the
475 presence of other membrane-associated proteins. No links between the TLR2
476 response, phylogenetic tree and the inferred ecological niches of the various strains
477 was either identified. This suggests that the TLR-2 response triggered by *L.*
478 *rhamnosus* is not reflected by the evolution of the species or its adaptation to one
479 particular niche, but is rather a trait acquired, maintained, altered or exacerbated by
480 other factors that remains yet to be identified.
481
482 **_L. rhamnosus_ vs. other bacterial populations**
483 *L. rhamnosus* isolates have been isolated from various ecological habitats, showing its
484 large ecological versatility. Niche-specialized strains have evolved by developing
485 distinctive metabolic traits, phage resistance system, stress-resistance mechanisms and
486 colonization traits (such as the production of pili) to efficiently persist in an ecological
487 habitat. However, the microbiota of habitats such as the human intestinal tract or the
488 vaginal cavity are rich and complex, consisting of many phylotypes [2, 52]. *L.*
489 *rhamnosus* strains may therefore compete with other bacterial species by producing
490 bacteriocins that prevent growth of other bacterial populations. In niches such as

cheese products, the diversity and richness of the microbiota is much lower, suggesting less competition [53]. When testing the anti-microbial activity of almost all the *L. rhamnosus* strains ($n = 92$) against pathogens *E. coli*, *Yersinia enterocolica* and *Listeria monocytogenes* at different pH, we found that most strains displayed anti-microbial activity (Additional Figure S6). This is in line with previous studies on *L. rhamnosus* anti-microbial activity [34, 54, 55]. Remarkably, most food isolates shared a similar profile and were clustered together, *i.e.* poor anti-microbial activity against *E. coli* and, to a lesser degree, against *L. monocytogenes*. The human strains, including LGG, had higher level of antimicrobial activity against the three human pathogens tested than most food strains. A high proportion of food isolates seems to have lost some abilities to produce antimicrobial compounds, suggesting that such trait might not be essential in a stable environment rich in nutrients and with lower microbiota diversity than in the intestinal tract. In contrast, the antagonistic assays revealed the fitness of human isolates to complex niches, where competing with other bacteria is essential to persist.

Species diversity, niche-adaptation and ecological dynamics

This study aimed at looking at the present of *L. rhamnosus*, *i.e.* genome *vs.* lifestyle *vs.* phenotype, but also at its past, to understand how the species *L. rhamnosus* evolved to be what it is now. The analysis of all 100 isolates clearly showed a duality in the evolution of *L. rhamnosus* species, as well at the genomic level as at the phenotypic level, with some traits typically associated to a specific niche.

Close inspection of the phylogenetic clustering of the 100 *L. rhamnosus* strains, based on their genome sequences, showed that this is paralleled by clustering of phenotypic data, including carbohydrate metabolism, antagonistic activity, resistance to bile salts

and pilosotype (Figures 1, 2, 3 and 4). In Figure 1, the cluster 1 contains *L. rhamnosus* strains that are mostly derived from food products and include the ones that can utilize lactose, indicating their adaptation to the dairy environment. In comparison with LGG, they underwent significant genome decay and rearrangements. The PTS and metabolic-related genes non-essential in cheese products were lost or decayed, *i.e.* loss of L-fucose utilization. In parallel, we hypothesized that additional functions were acquired possibly through horizontal gene transfer, genetic mobile elements or plasmids, *i.e.* the ability to use lactose, a major carbon source in milk-derivative products. The clear changes of fermentative profiles (Figure 2) along with genome adaptation, illustrates how the strains evolved in different habitats. The loss of pili in these food strains is another characteristic example of a trait lost during niche-adaptation, where the absence of mucosa surfaces is reflected by the decay or complete loss of the non-essential pili. In the cheese or milk niche, phage predation is ubiquitous as showed in many LAB studies [56, 57]. Therefore, the CRISPR system might evolve by the acquisition of spacers representative of phages or plasmids of a particular niche. This is the case as the CRISPR locus profile of food isolates differ considerably from that in LGG. It is noteworthy that food isolates have a diverse resistance to bile salts, as discussed below. Opposite branches (clusters 3 and 4) include strains that are highly similar to LGG in terms of genome content (Figure 1). Most of them were isolated from human cavities. These strains present similar fermentative profiles and CRISPR spacer oligotypes with only subtle differences, suggesting that these strains share close ancestor with LGG but are not *L. rhamnosus* LGG.

A detailed analysis of the species revealed how some subgroups evolved in one or multiple niches. When first looking at the intestinal tract isolates, typically, two

distinct populations could be observed among them (Figure 6). The first population group showed a high similarity with LGG in terms of genomes and phenotypes. They produced mucus-binding pili structures, promoting the colonization of the human intestine and the interaction with the host cells, and are also resistant to the bile salts. These lifestyle traits confer them adequate fitness to the intestinal tract, suggesting that these strains are well adapted to this. In contrast, the second group of *L. rhamnosus* strains is more genetically and phenotypically related to food-specialized strains that are characterized by a lack of pili, a different carbohydrate metabolism and a distinct CRISPR system profile. This indicates that these isolates were likely introduced in the intestinal tract *via* consumption of foods. Due to their bile resistance, they were able to survive in the intestinal tract but may not be able to compete with other autochthonous gut bacteria to colonize the intestinal tract as they lack the mucus-binding pili. We propose that most of these isolates were in transit in the intestinal tract and further eliminated along with the faecal material (Figure 6). Other *L. rhamnosus* food isolates that are bile sensitive may also be introduced in the gastro-intestinal tract *via* the diet but cannot survive the intestinal conditions. Interestingly, *L. rhamnosus* from the vaginal cavity and urethra show a very similar phenotype/genotype as these 'in-transit' *L. rhamnosus* strains isolated from the intestinal tract, which is in agreement with previous studies showing that the rectal microbiota is a potential reservoir of bacteria that may colonize the vaginal cavity [50]. Most vaginal isolates are more related to the 'in-transit' isolates (Figure 6), suggesting that the 'in-transit' isolates may be more adapted to the vaginal environment, possibly due to their distinct metabolic abilities. This however remains speculative, as at individual level, we do not know which *L. rhamnosus* strains these women possibly have in the intestinal tract. Most *L. rhamnosus* strains used in

566 probiotic products are known to originate from the human intestinal tract, which
567 concords with our findings, as they are very similar to the 'permanent' residents from
568 the intestinal tract. This also indicates that the intestinal tract is a potential reservoir
569 for new candidates for use in probiotic products, provided that they are not
570 passengers. Regarding the isolates from the oral cavity, the results of the metabolic
571 profiling indicate that they likely originated from the diet. Due to the low number of
572 strains, it is however difficult to draw any definitive conclusions. The last and largest
573 group of blood and clinical isolates is a very diverse pool of isolates, representative of
574 the whole species. No specific patterns of adaptation were found at genome and
575 phenotype level. The ephemeral presence of *L. rhamnosus* in these niches therefore
576 cannot be reflected in its genetic and phenotypic traits. Although some of these
577 isolates had similar gene content as LGG, metabolic profiles and CRISPR spacer
578 oligotyping clearly show that these strains are not identical to LGG. This is in line
579 with a previous study that showed that the widespread and increasing use of probiotic
580 strain LGG was not associated with the augmentation of *Lactobacillus* bacteremia
581 [26]. A very good correlation between the metabolic profiling, CRISPR sequences
582 and pilosotypes was observed, suggesting that the use of these basic and
583 complementary analyses might be sufficient to identify the origin of the *L. rhamnosus*
584 strains.
585 Genomic and functional analysis is a powerful approach to understand how bacteria
586 evolved and also provide some information about the history of different isolates
587 (Figure 6). For example, dairy strain F0962 is of particular interest, due to its high
588 genetic relatedness with LGG (Figure 1). Virtually syntenous to LGG, strain F0962
589 genome underwent some gene decays, since it does not use L-fucose, suggesting that
590 the fucose transport and metabolism is defective in F0962. When tested for the use of

591 other carbohydrates, F0962 presents a similar fermentative profile as other food
592 isolates, *i.e.* capability of metabolizing D-lactose, D-maltose, and L-rhamnose. It is
593 bile resistant and piliated, as confirmed by immunoblotting analysis and mucus
594 binding. This indicates that strain F0962 may be originally from the intestinal tract
595 and might have been recently introduced into a fermentation environment and evolved
596 in its new niche by possibly decaying some of its non-essential genes. Evolution-wise,
597 that strain might further specialize and lose more genes, as well as acquire novel
598 biological functions by persisting in the same niche.
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Conclusions

The analysis of the genomes and phenotypes of 100 strains of the species *L. rhamnosus* provided a wealth of information with respect to the genetic traits that are essential in different ecological niches and how the species *L. rhamnosus* evolved. The variable regions that we observed in the *L. rhamnosus* genomes are good markers of the species evolution and adaptation to various niches (Figure 6) and allowed us to gain insights on the past of each strain, including its dynamics and ecological fitness. The present study also calls attention to the genome stability of *L. rhamnosus*, since some intestinal isolates are widely used in dairy industry. Domestication of lactic acid bacteria isolated from human cavities is usually accompanied by important genome alteration, causing the loss of lifestyle islands [31]. In *L. rhamnosus*, we clearly observed that the food isolates had undergone major genome decay, resulting in different metabolic capabilities, stress resistance and host interaction that could be associated with unstable chromosomal regions rich in transposases, *i.e.* SpaCBA pili gene cluster. Therefore, the phenotypic and genotypic traits highlighted in this study may be valuable to understand the ecology of novel *L. rhamnosus* isolates, to identify novel probiotic candidates and also to closely monitor the genome stability and functional properties of current commercial *L. rhamnosus* strains.

639 ## Methods

640 ### *L. rhamnosus* isolate collection, DNA isolation and molecular typing

641 All *Lactobacillus rhamnosus* strains used in this study were obtained from various
642 institutions, universities and hospitals (Table S1). A total of 100 strains were
643 analysed, 71 of human origin and 29 of food origin. Well-characterized, *L. rhamnosus*
644 GG was used as reference strain throughout the study [7, 11, 33]. Strains VIFIT,
645 IDOF, AKRO, CORO and NEO-IM were isolated from probiotic-marketed products
646 (Table S1), whereas a number of strains were made available from strain collections.
647 Information relative to the entire *L. rhamnosus* bacterial isolate collection used in this
648 study is shown in Additional Table S1. All isolates were routinely propagated in
649 anaerobic conditions at 37°C in MRS medium (Difco BD, NJ, USA). Chromosomal
650 DNA from each isolate was extracted using Wizard Genomic DNA Purification Kit
651 (Promega, WI, USA) following the manufacturer's instructions. Initial bacterial
652 identification at the species level was performed by amplification of *tuf* gene as
653 described by Ventura *et al*. [58, 59] using standard PCR amplification conditions and
654 multiplex PCR amplification (data not shown).
655
656 ### Fermentative profile

657 The sugar metabolism and other catabolic properties of the *L. rhamnosus* strains were
658 investigated using API CH 50 kit (bioMerieux, Marcy L'Etoile, France). All strains
659 were grown until logarithmic phase and then inoculated in API galleries following the
660 manufacturer's instructions. API galleries were further incubated at 37°C in anaerobic
661 conditions for 48 h prior to colorimetric analysis.
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Genome SOLiD sequencing and bioinformatic sequence analysis

Genomes of all *L. rhamnosus* isolates were sequenced on a SOLiD sequencer platform (Life Technologies) at the Institute of Biotechnology (Helsinki, Finland). Sequence alignments and consensus sequences were generated by mapping SOLiD color space reads to LGG genome as the reference genome, using the SOLiD BioScope software (Life Technologies) and the SAM tools (Li *et al*., 2009). In order to transfer annotation from a reference genome (*L. rhamnosus* GG) to each un-annotated mapped genome, sequences were compared with 'nucmer' to identify regions that share synteny [60]. Those regions were extracted as base range in the mapped genome and in the reference genome (LGG). In-house custom-made scripts were then used to transfer annotation. Synteny blocks had a nucleotide sequence identity more than or equal to 40%. For each query genome, a set of shared LGG orthologous genes was obtained and further analyzed. LGG genome was assigned to COGs using Reverse Position Specific blast and Conserved Domain Database from NCBI. Mapped genome sequences may be available upon request.

Human mucus binding assay

Mucus binding adhesion assays were performed as previously described [11, 61]. Briefly, MaxiSorp microtiter plates (Nunc, Denmark) were coated with 100 µL of human mucus solution prepared in PBS at a final concentration of 0.5 mg/mL and further incubated overnight at 4°C. The wells were then washed with PBS to remove unbound mucus and 100 µL of ^{3}H-radiolabeled bacterial suspensions at optical density (OD_{600}) 0.25±0.01 were added to the wells. The microtiter plate was further incubated at 37°C for 1h and then wells were washed with PBS in order to remove unbound bacteria. Bacteria adhering to mucus were incubated at 60°C for 1h in 1%

689 SDS-0.1 M NaOH solution and the radioactivity level of lyzed bacterial suspensions
690 was measured by liquid scintillation counting in a Wallac 1414 liquid scintillation
691 counter (PerkinElmer). The percentage ratio between radioactivity values of lysed
692 bacteria suspension and bacteria suspension added initially to the well indicated the
693 adhesion to intestinal mucus. For each strain the experiment was performed in
694 quadruplicate.
695
696 **Antiserum-mediated human mucus binding assay**
697 Human mucus binding assay was performed for *L. rhamnosus* isolates in the presence
698 of polyclonal SpaC antibody as described above. ^{3}H radio radiolabeled bacteria were
699 co-incubated with the immobilized mucus in the presence of a 1:100 dilution of anti-
700 SpaC serum.
701
702 **Immunoblotting analysis of cell wall proteins**
703 For each isolate, bacterial suspension adjusted to an optical density (OD_{600}) of 1.0 was
704 used to extract cell wall-associated proteins. Cell pellets were washed once with PBS
705 and disrupted mechanically by bead-beating using sterile quartz beads (Merck KGaA,
706 Germany). Cell wall material was resuspended in 500 µL of PBS and further pelleted
707 by centrifugation at high speed for 30 min. Next, the samples were digested for 3 h at
708 37°C in a 50 µL enzymatic mixture containing 50mM Tris-HCl, 5mM MgCl2, 5mM
709 CaCl2, 10mg/mL lysozyme and 150 U/mL mutanolysin. Samples were mixed with
710 12.5 µL of 4X Laemmli loading buffer (BioRad, CA, USA) and heated at 99°C for 10
711 min. Cell wall proteins were resolved on 10% acrylamide gel and electroblotted onto
712 0.2 µm nitrocellulose membrane (BioRad, CA, USA). Polyclonal rabbit SpaA
713 antiserum (1:10,000) and peroxidase-conjugated goat anti-rabbit IgG (Jackson

ImmunoResearch, USA) (1:10,000) were respectively used as a primary and secondary antibody in 5% fat-free milk/PBS solution. Membranes were blocked with 5% fat-free milk/PBS solution, and washed with 0.05% Tween 20– PBS solution in-between incubations. Membranes were analyzed using the in-house electrochemiluminescent method.

Detection of Secreted Embryonic Alkaline Phosphatase (SEAP)

HEK-blueTM hTLR2/4/5 cell lines (Invivogen, CA, USA) were used in this assay. All cell lines were grown and subcultured up to 70-80% of confluency using as a maintenance medium Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5 g/L glucose, 50 U/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL µg/mL NormocinTM, 2mM L-glutamine, and 10% v/v of heat-inactivated fetal bovine serum. For each cell line, the immune response assay was carried out by splitting HEK-blueTM cells in flat-bottom 96-well plates and stimulating them by addiction of bacterial suspension adjusted to OD_{600} 1, 1:10, 1:100. The 96-well plates were incubated for 20-24 h at 37°C in a 5% CO2 incubator. Receptor ligands as PAM3CSKA (100ng/mL for hTLR2), LPS-EB (100ng/mL for hTLR4) and RecFLA-ST (10ng/mL for hTLR5) were used as positive control while maintenance medium without any selective antibiotics was used as negative control. SEAP secretion was detected by measuring the OD600 at 15min, 1h, 2h, and 3h after addition of 180 µL of QUANTI-BlueTM media (Invivogen, CA, USA) to 20µL of induced HEK-blue^{TM} hTLR2/4/5 supernatant. All cell lines were stimulated in triplicate for each isolate.

TEM Sample Preparation.

Selected *L. rhamnosus* isolates were analyzed by transmission electron microscopy (TEM) as previously described by Reunanen *et al*. [12]. Briefly, 20 µL of overnight bacterial cultures were added to Formvar-carbon-coated copper grids for 30 min at room temperature. Grids were then washed three times with 0.02 M glycine solution and further incubated for 15 min in a blocking solution containing 1% w/v of bovin serum albumin (BSA). Next, a 1:100 dilution of SpaA antibody was prepared in 1% w/v BSA solution and added to the grids for 1h, washed with 0.1% w/v BSA and incubated for 20 min with protein A conjugated to 10 nm gold particles. Grids were washed several times in PBS, fixed for 5 min using 1% glutaraldehyde, washed again with deionized water and stained with a solution containing 1.8% methycellulose and 0.4% uranyl acetate. Grids were visualized using JEOL 1200 EX II transmission electron microscope (JEOL Ltd., Japan).

Bile resistance assay

L. rhamnosus strains were cultured in MRS broth at 37°C in anaerobic conditions. The OD_{600} of the bacterial culture suspensions were equalized. 3 µl of cell suspensions were spotted onto MRS agar plates containing 0.5 % w/v Ox gall bile salts (Sigma, MO, USA). Plates were incubated anaerobically at 37°C for two days and visually examined.

Antagonistic assay

L. rhamnosus strains were grown until stationary phase as described above. Next, the cell suspensions were thoroughly homogenized and the OD_{600} was equalized. Cell mixtures were then centrifuged for 20 min at 650 x g at +5°C and the supernatants were pH-adjusted at 5 and 6.20 by addition of NaOH and HCl solutions, filtered (0.22

763 µm filter) and stored at-20°C for further analysis. Antagonistic assays were
764 performed in microtiter well plate with a turbidometric assay as previously described
765 [62]. *E. coli* O157 (ATCC 43894), *L. monocytogenes* R14-2-2 (DVME) and *Y.*
766 *enterocolitica* R5-9-1 (DVME) were incubated for 15 h at 37°C in the presence of 20
767 µl of *L. rhamnosus* pH-adjusted supernatant. The OD_{600} values were measured in an
768 automatic reader (Bioscreen C, Oy Growth Curves Ab Ltd, Finland) every 30 min,
769 and the bacterial growth was quantified using growth curves and the area under curve
770 (AUC) values, automatically processed by the BioLink software (Oy Gorwth Curves
771 Ab), and Inhibition was expressed as an area reduction percentage (ARP) compared to
772 control samples grown without the addition of supernatant .
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Authors' contributions

FPD designed the study, wrote the manuscript, analyzed the data and performed some mucus-binding, immunoblotting analyses, bile assays and API tests. AR designed the study, isolated some of the strains, extracted DNA for genome sequencing, performed mucus-binding assays, API tests, immunoblotting analyses, bile assays, antagonistic assays and immunoassays and, drafted the manuscript. RK designed the study, performed bioinformatics analysis and drafted the manuscript. HMJ and MM extracted some genomic DNA for genome sequencing and performed some immunoblotting analyses. TEP participated in immunoassays. CLR and CC isolated some of the *L. rhamnosus* strains. LP conducted the genome sequencing, performed some post-sequencing data analysis and drafted the manuscript. PL and JAR performed SOLiD data assembly and mapping. RS participated to mucus binging assays. SB was involved in the CRISPR analysis. TL participated in antagonistic assays. IvO produced and supplied anti-SpaA and anti-SpaC pilin sera for use in immunoblotting, mucus adhesion, and TEM analyses. JR performed the TEM analysis and participated in the immunoblotting analysis. AP co-supervised the study. WMV designed the study, supervised the entire study and drafted the manuscript.

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815 Competing interests

816 The authors of the present manuscript have declared that no competing interest exists.

817 Acknowledgements

818 This study was supported by the grant ERC 250172 - Microbes Inside from the
819 European Research Council, the Center of Excellence in Microbial Food Safety
820 Research (CoE-MiFoSa), Academy of Finland (grant 141140) and the University of
821 Helsinki. François P. Douillard was also financially supported by a postdoctoral
822 research fellowship from the Academy of Finland (grant 252123). We thanked Pia
823 Rasinkangas, Edward Alatalo and Okeke Godfrey Uche for technical assistance. We
824 are very grateful to Prof Paul Ross and Prof Gerald Fitzgerald (TEAGASC, UCC and
825 the Alimentary Pharmabiotic Centre, Cork, Ireland), Dr Soile Tynkkynen (Valio Ltd.,
826 Helsinki, Finland), Prof Remco Kort (TNO, Zeist, The Netherlands), Prof Neviani
827 Erasmo (Department of Food Science, University of Parma, Italy) and Prof Giovanni
828 Furnari (Department of Biomedical Sciences, Microbiology Section, University of
829 Catania, Italy) for generously providing some of the *L. rhamnosus* strains. We thank
830 Prof Seppo Salminen (Functional Foods Forum, Turku, Finland), Dr Satu Vesterlund
831 (University of Turku, Finalnd) and MD Heikki Huhtinen (Turku University Central
832 Hospital, Turku, Finland) for collecting and providing the human intestinal mucus
833 samples.

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Figures

Figure 1: Analysis of genome diversity in *L. rhamnosus* by mapped SOLiD sequencing.

Panel (A) shows the phylogenetic tree of 100 *L. rhamnosus* strains based on their relative shared gene content with *L. rhamnosus* GG. The tree branches have been coloured and numbered to highlight the main sublineages. Colour code: green for food origin, red for human origin and pink for probiotic-marketed product origin. Panel (B) indicates the percentage of shared gene content with LGG for each strain.

Figure 2: API 50CH fermentative profile of *L. rhamnosus* strains.

Fermentation ability is indicated in black for positive, grey for partially positive and white for negative. Strains are organized according to their genetic relatedness as defined in the phylogenetic tree and coloured according to their respective sublineage (shown in Figure 1). Carbohydrates of particular interest are marked with a red asterisk. Black arrows show fermentative profile shifts among *L. rhamnosus* strains.

Figure 3: CRISPR-associated protein diversity and CRISPR spacer oligotyping in *L. rhamnosus* species.

Panel (A) illustrates the genetic organization of the CRISPR system and its associated genes in LGG. Panel (B) shows the conservation (blue), the partial conservation (grey) or the absence (yellow) of LGG spacers. The presence (white) or the absence (black) of the *cas* genes is also indicated at the bottom of the panel. Strains are organized according to their genetic relatedness shown in Figure 1.

Figure 4: Bile resistance is associated with particular niches.

Strains were classified as resistant, moderately resistant, poorly resistant or sensitive to bile salts. Results were grouped per niches. The table attached to the plot indicates the number of strains in each niche.

Figure 5: Mucus adhesion and SpaCBA pili gene diversity among *L. rhamnosus*.

Panel (A) shows the human mucus binding ability (%) of all *L. rhamnosus* isolates ranked from the lowest to the highest mucus binder. Panel (B) shows the genotype and phenotype of all strains. In the genotype part were compiled data from our large genomic analysis, where pilin and sortase genes are assigned as present (green) or divergent (red). In addition, sequences of corresponding genes were further analyzed by blastx. The sequence identity was shown by a triangle superposed to the SOLiD data, where the colour gradient corresponds to the percentage of identity to LGG genes as indicated in the figure. In phenotypes are indicated if the strains were tested by immunoblotting analysis (DB), electron microscopy (EM) and *in vitro* competitive binding assay (AB). Green is for pili positive and red for pili negative.

Figure 6: Genome diversity in *L. rhamnosus* reveals strain adaptation to a given ecological niche.

It relies on gene acquisition and loss, point mutations, genetic reorganization, bacteriophages and plasmids. Such genetic events promote adaptability of a strain to a new ecological niche. For each niche, the most representative persistence traits are shown.

1111 # Tables

1112 **Table 1: Features of the variable chromosomal regions found in *L. rhamnosus*.**

1113 Variable regions were numbered 1 to 17 and mentioned accordingly in the main text.

Region	Genes	GI	IS	Main genetic features of the region
1	LGG_00170-LGG_00177	-	-	taurine ABC transporter, conserved protein, transcriptional regulator
2	LGG_00278-LGG_00283	-	-	rhamnosyl PTS, rhamnosyltransferase
3	LGG_00341-LGG_00347	-	-	galactitol PTS, conserved protein
4	LGG_00376-LGG_00427	1	2 IS	transcriptional regulator, hypothetical protein, fructose PTS, lactose PTS, mannose PTS, conserved protein,
5	LGG_00438-LGG_00481	2	11 IS	conserved protein, SpaCBA pili cluster, transcriptional regulator, ABC transporter
6	LGG_00511-LGG_00517	-	2 IS	ABC transporter, conserved protein
7	LGG_00559-LGG_00566	-	-	conserved protein, transporter, sugar phosphate isomerase
8	LGG_01023-LGG_01029	-	3 IS	restriction/modification enzymes
9	LGG_01086-LGG_01143	3	-	conserved protein, phage-related protein
10	LGG_01515-LGG_01544	4	1 IS	phage-related protein, conserved protein
11	LGG_01955-LGG_01967	-	5 IS	conserved protein
12	LGG_01990-LGG_02003	-	1 IS	conserved protein, UDP-N-acetylglucosamine 2-epimerase, lyzozyme
13	LGG_02038-LGG_02056	5	1 IS	EPS cluster
14	LGG_02199-LGG_02204	-	-	CRISPR-associated genes, CRISPR
15	LGG_02610-LGG_02614	-	-	ABC transporter, conserved protein
16	LGG_02651-LGG_02686	-	1 IS	fucose transporter, conserved protein, transcriptional regulator
17	LGG_02742-LGG_02755	-	1 IS	conserved protein, Fructose-bisphosphate aldolase, mannose/fructose/lactose PTS, galactitol PTS

Table 2: Pilosotype distribution in the *L. rhamnosus* collection used in the study.

Are described the niches, the number of strains per niche and their pilosotype, *i.e.* the presence of an intact and functional SpaCBA pili cluster.

Sources	SpaCBA positive	SpaCBA negative	Total	% SpaCBA
Human	**29**	**43**	**72**	**40**
Blood	14	19	33	50
Vaginal cavity /urethra	0	8	8	0
Oral Cavity	0	3	3	0
Intestinal tract	7	5	12	58
Others	8	8	16	50
Food	**5**	**23**	**28**	**18**
Parmigiano Regiano cheese	3	9	12	25
Pecorino cheese	0	9	9	0
Probiotic-marketed products	2	3	5	40
Other cheeses	0	2	2	0
	34	**66**	**100**	**35**

Additional files

Additional Table S1: List of *L. rhamnosus* strains used in the present study.

Strains have been obtained or isolated from various institutions and labelled as follows: FIN-U for Department of Veterinary Medicine, Helsinki University, Finland; FIN-V for Valio Ltd., Helsinki, Finland; ITA-C for Department of Microbiology and Food Technology, University of Catania, Italy; ITA-F for Department of Bio-Medical Sciences, Microbiology section, University of Catania, Italy; ITA-P for Department of Genetics, Biology of Microorganisms, Anthropology, Evolution, University of Parma, Parma, Italy; IRL for TEAGASC & Alimentary Pharmabiotic Centre, UCC, Cork, Ireland and NL-Y for Yoba for Life Foundation, Amsterdam, The Netherlands. Strains obtained from Valio Culture Collection Ltd. were initially isolated and collected by the HUSLAB (Helsinki University Central Hospital Laboratory, Helsinki) and other clinical laboratories around Finland.

Additional Table S2: List of LGG non-core genes

The core genome of *L. rhamnosus* can be deducted from the present gene list.

Additional Table S3: Comparative genomic data of 100 *L. rhamnosus* strains.

Legend: 1 for gene present in that particular strain and 0 for divergent/missing gene.

Additional Table S4: BLAST analysis of the spacers present in LGG CRISPR locus.

Each spacer was blasted against the virus and plasmid sequence database using sensitive BlastN setting: word size 7, expected threshold 0.1, match/mismatch 1,-1.

Additional Figure S1: COG distribution in *L. rhamnosus* shared gene subset, LGG genome and LGG-specific gene subset.

Additional Figure S2: Overview of the 17 variable regions reported in 100 *L. rhamnosus* strains.

The frequency of gene loss was calculated for each LGG gene and plotted on the X-axis that represents LGG chromosome. Each numbered region is described in Table 2. In addition, other regions were labelled as follows: i for IS elements, ii for conserved proteins, iii for metabolism-associated genes.

Additional Figure S3: Adhesion of *L. rhamnosus* strains to human mucus in the presence of SpaC anti-serum.

Radiolabeled (^{3}H) cells of 22 different *L. rhamnosus* isolates were tested in the presence or the absence of polyclonal antibodies directed against SpaC pilin subunit. The assay was performed in triplicates.

Additional Figure S4: Electron microscopy observation of pili in *L. rhamnosus* strains using immunogold staining.

Ten *L. rhamnosus* strains were labelled with anti-SpaA gold particles (10 nm diameter) and observed by electron microscopy. Black arrows indicate pili structures. Black bar represents 0.5 µm. Legend: A for LGG; B for H1249; C for H1242; D for H1031; E for H1094; F for H1180; G for H1101; H for H1102; I for H1225; J for H1129.

Additional Figure S5: Response of HEK-Blue™ hTLR2 cell line to various *L. rhamnosus* strains.

HEK-Blue™ hTLR2 cells were co-incubated with one of the *L. rhamnosus* strains for 24h. NF-κB-induced SEAP activity was further quantified by spectrophotometry. The data are expressed as means ± standard deviation.

Additional Figure S6: Anti-microbial activity of *L. rhamnosus* strains against *E. coli*, *Yersina enterocolica* and *Listeria monocytogenes*.

Ninety two *L. rhamnosus* strains were tested for potential anti-microbial activity as described in the Methods section. The filtrates used in the experiment were adjusted at two different pH: 5.0 and 6.2. Colour legend for the heat map: green for significant anti-microbial activity, black for no activity and red for inverse effect. The color scale used for the heat-map is ranging from -10% to +10%. Colour legend for the strains: green for food strains, pink for probiotic-marketed strains and red for human strains.

1202 **Fig.1**

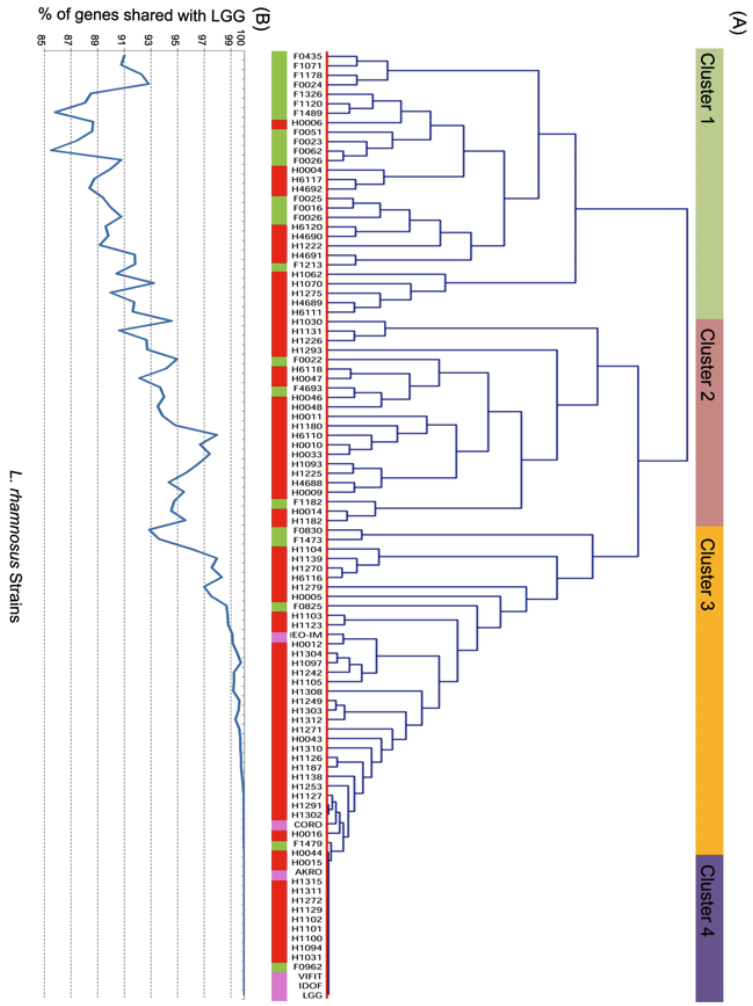

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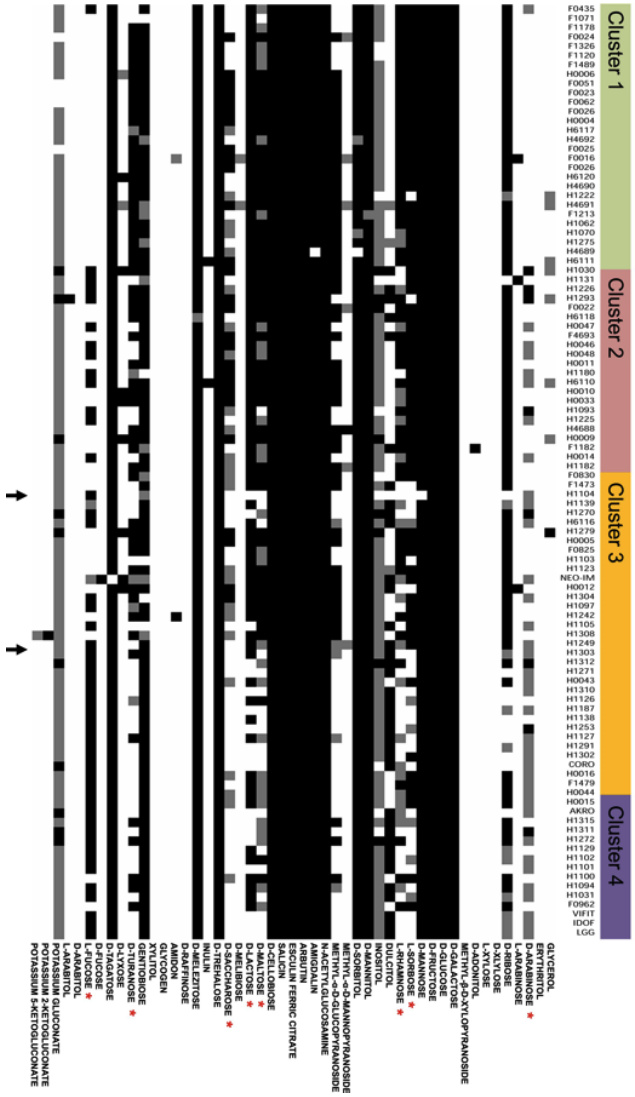
Fig.2


Cluster 1
Cluster 2
Cluster 3
Cluster 4
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ERYTHRITOL
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L-ARABINOSE *
D-RIBOSE
D-XYLOSE
L-XYLOSE
D-ADONITOL
METHYL-βD-XYLOPYRANOSIDE
D-GALACTOSE
D-GLUCOSE
D-FRUCTOSE
D-MANNOSE
L-SORBOSE *
L-RHAMNOSE *
DULCITOL
INOSITOL
D-MANNITOL
D-SORBITOL
METHYL-αD-MANNOPYRANOSIDE
METHYL-αD-GLUCOPYRANOSIDE
N-ACETYLGLUCOSAMINE
AMIGDALIN
ARBUTIN
ESCULIN FERRIC CITRATE
SALICIN
D-CELLOBIOSE
D-MALTOSE *
D-LACTOSE *
D-MELIBIOSE *
D-SACCHAROSE *
D-TREHALOSE
INULIN
D-MELEZITOSE
D-RAFFINOSE
AMIDON
GLYCOGEN
XYLITOL
GENTIOBIOSE
D-TURANOSE *
D-LYXOSE *
D-TAGATOSE
D-FUCOSE
L-FUCOSE *
D-ARABITOL
L-ARABITOL
POTASSIUM GLUCONATE
POTASSIUM 2-KETOGLUCONATE
POTASSIUM 5-KETOGLUCONATE
F0435
F1071
F1178
F0024
F1326
F1120
F1489
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F0051
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F0062
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H1127
H1291
H1302
CORO
H0016
F1479
H0044
H0015
AKRO
H1315
H1311
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F0962
VIFIT
IDOF
LGG

1212 **Fig.3**

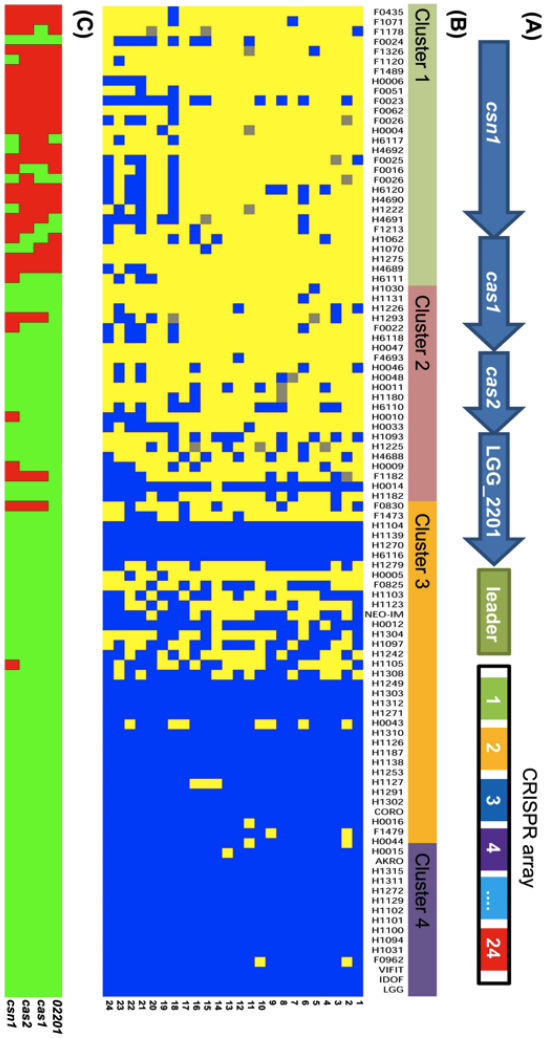

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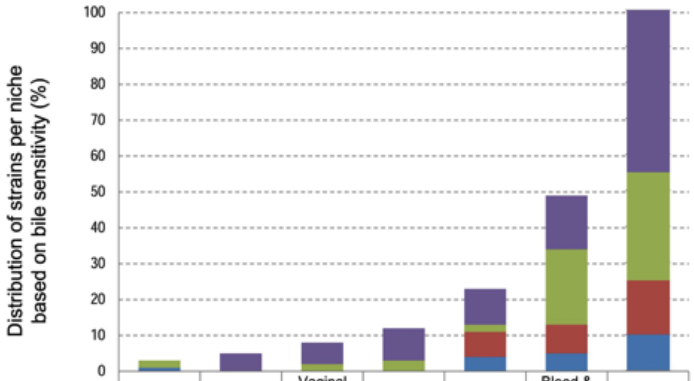
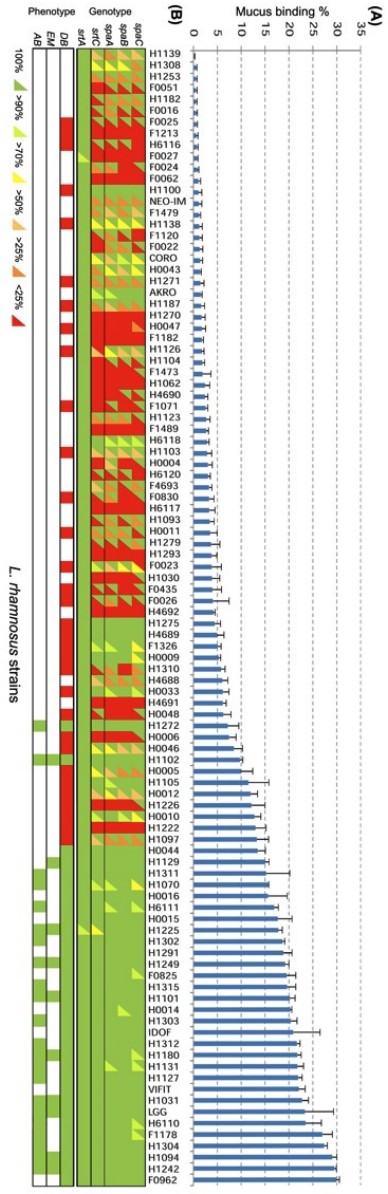
Fig.4



	Oral cavity	Probiotics	Vaginal cavity & urethra	Intestinal tract	Cheese	Blood & Clinical isolates	Total
Resistant	0	5	6	9	10	15	45
Moderately resistant	2	0	2	3	2	21	30
Poorly resistant	0	0	0	0	7	8	15
Sensitive	1	0	0	0	4	5	10

1232 **Fig.5**



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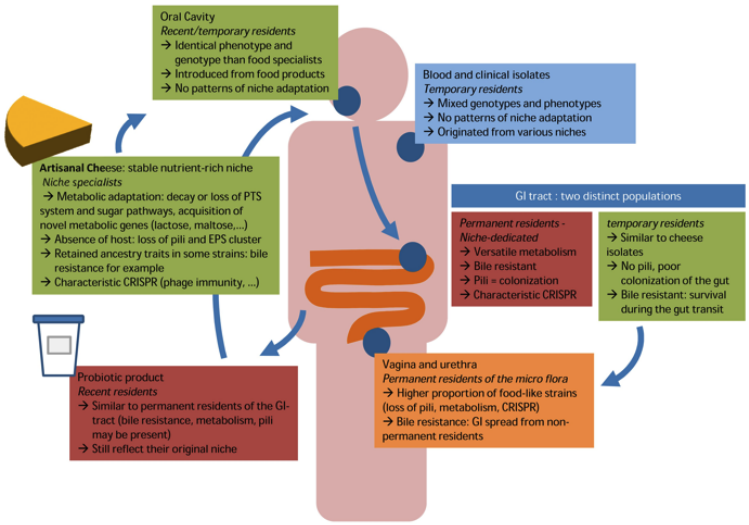
1234 **Fig.6**



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1249 **Additional Table S1**

#	Strain Name	Names used in the study	Source	Institute
1	AK-RO-01	AKRO	Yoba yogurt	NL-Y
2	CO-RO-01	CORO	Yoba yogurt	NL-Y
3	D16	F0016	Pecorino cheese (60 days)	ITA-C
4	D22	F0022	Pecorino cheese (60 days)	ITA-C
5	D24	F0023	Pecorino cheese (60 days)	ITA-C
6	E24	F0024	Pecorino cheese (60 days)	ITA-C
7	H25	F0025	Pecorino cheese (120 days)	ITA-C
8	E26	F0026	Pecorino cheese (60 days)	ITA-C
9	D26	F0027	Pecorino cheese (60 days)	ITA-C
10	H51	F0051	Pecorino cheese (120 days)	ITA-C
11	E62	F0062	Pecorino cheese (60 days)	ITA-C
12	435	F0435	Panerone cheese	ITA-P
13	825	F0825	Parmigiano Reggiano curd	ITA-P
14	830	F0830	Parmigiano Reggiano curd	ITA-P
15	962	F0962	Parmigiano Reggiano cheese (90 days)	ITA-P
16	1071	F1071	Parmigiano Reggiano cheese (8 months)	ITA-P
17	1120	F1120	Parmigiano Reggiano cheese (10 months)	ITA-P
18	1178	F1178	Parmigiano Reggiano cheese (12 months)	ITA-P
19	1182	F1182	Parmigiano Reggiano cheese (12 months)	ITA-P
20	1213	F1213	Parmigiano Reggiano cheese (12 months)	ITA-P
21	1326	F1326	Parmigiano Reggiano cheese (16 months)	ITA-P
22	1473	F1473	Parmigiano Reggiano cheese (20 months)	ITA-P
23	1479	F1479	Parmigiano Reggiano cheese (20 months)	ITA-P
24	1489	F1489	Parmigiano Reggiano cheese (20 months)	ITA-P
25	APC 4693 (LMG 12166)	F4693	hard cheese	IRL
26	Lac 4	H0004	vaginal cavity	ITA-F
27	Lac 5	H0005	vaginal cavity	ITA-F
28	Lac 6	H0006	vaginal cavity	ITA-F
29	Lac 9	H0009	vaginal cavity	ITA-F
30	Lac 10	H0010	vaginal cavity	ITA-F
31	Lac 11	H0011	vaginal cavity	ITA-F
32	Lac 12	H0012	vaginal cavity	ITA-F
33	E14Cork	H0014	intestinal tract	IRL
34	E16a	H0015	intestinal tract	IRL
35	E16b	H0016	intestinal tract	IRL
36	Lac 33	H0033	oral cavity	ITA-F
37	E43Cork	H0043	intestinal tract	IRL
38	E44Cork	H0044	intestinal tract	IRL

#	Strain Name	Names used in the study	Source	Institute
39	Lac 46	H0046	oral cavity	ITA-F
40	E47 Cork	H0047	clinical isolate	IRL
41	Lac 48	H0048	oral cavity	ITA-F
42	1030 (TT 4813)	H1030	blood	FIN-V
43	1031 (TT 4846)	H1031	blood	FIN-V
44	1062 (TT 24029)	H1062	blood	FIN-V
45	1070 (TT 25865)	H1070	blood	FIN-V
46	1093 (TT 23808)	H1093	blood	FIN-V
47	1094 (TT 70980)	H1094	blood	FIN-V
48	1097 (TT 70977)	H1097	blood	FIN-V
49	1100 (TT 71004)	H1100	blood	FIN-V
50	1101 (TT 71005)	H1101	blood	FIN-V
51	1102 (TT 71006)	H1102	blood	FIN-V
52	1103 (TT 71007)	H1103	blood	FIN-V
53	1104 (TT 71009)	H1104	blood	FIN-V
54	1105 (TT 71034)	H1105	Abscess	FIN-V
55	1123 (TT 71273)	H1123	clinical isolate	FIN-V
56	1126 (TT 71311)	H1126	clinical isolate	FIN-V
57	1127 (TT 71326)	H1127	clinical isolate	FIN-V
58	1129 (TT 71330)	H1129	clinical isolate	FIN-V
59	1131 (MES 8296)	H1131	pus	FIN-V
60	1138 (TT 32154)	H1138	clinical isolate	FIN-V
61	1139 (TT 71353)	H1139	blood	FIN-V
62	1180 (TT 33620)	H1180	blood	FIN-V
63	1182V	H1182	clinical isolate	FIN-V
64	1187 (TT 33651)	H1187	blood	FIN-V
65	1222 (TT 15756)	H1222	blood	FIN-V
66	1225 (TT 21162)	H1225	blood	FIN-V
67	1226 (TT 19557)	H1226	blood	FIN-V
68	1242 (TT 36186)	H1242	blood	FIN-V
69	1249 (TT 38522)	H1249	blood	FIN-V
70	1253 (TT 72663)	H1253	blood	FIN-V
71	1270 (TT 73573)	H1270	clinical isolate	FIN-V
72	1271 (TT 73572)	H1271	clinical isolate	FIN-V
73	1272 (TT 38983)	H1272	blood	FIN-V
74	1275 (TT 39685)	H1275	blood	FIN-V
75	1279 (TT 41773)	H1279	blood	FIN-V
76	1291 (TT 74230)	H1291	blood	FIN-V
77	1293 (TT 74232)	H1293	blood	FIN-V
78	1302 (TT 74293)	H1302	clinical isolate	FIN-V
79	1303 (TT 74294)	H1303	clinical isolate	FIN-V
80	1304 (TT 74236)	H1304	clinical isolate	FIN-V
81	1308 (TT 42258)	H1308	blood	FIN-V

#	Strain Name	Names used in the study	Source	Institute
82	1310 (JCM 43966)	H1310	blood	FIN-V
83	1311 (JCM 42376)	H1311	blood	FIN-V
84	1312 (JCM 74518)	H1312	blood	FIN-V
85	1315 (JCM 74493)	H1315	clinical isolate	FIN-V
86	APC 4688 (CCUG23641)	H4688	blood	IRL
87	APC 4689 (CCUG27363)	H4689	hip punction	IRL
88	APC 4690 (LMG6400a)	H4690	blood	IRL
89	APC 4691 (LMG6400b)	H4691	blood	IRL
90	APC 4692 (LMG 8153)	H4692	healthy adult female urethra	IRL
91	APC 6110	H6110	infant isolate	IRL
92	APC 6111	H6111	infant isolate	IRL
93	APC 6116	H6116	infant isolate	IRL
94	APC 6117	H6117	infant isolate	IRL
95	APC 6118	H6118	infant isolate	IRL
96	APC 6120	H6120	infant isolate	IRL
97	Idoform LGG	IDOF	pharmaceutical product	FIN-U
98	*L. rhamnosus* LGG	LGG	intestinal tract, used in dairy products	FIN-U
99	Neo-Imunele	NEO-IM	probiotic-marketed yogurt	FIN-U
100	Vifit LGG	VIFIT	probiotic-marketed drinkable yogurt	FIN-U

1263 **Additional Table S2**

Gene Name	Symbol	Predicted Gene Product
LGG_00009	LGG_00009	Putative protein without homology
LGG_00020	is1	Transposase, IS30 family protein
LGG_00022	is2	Transposase, IS5 family protein
LGG_00023	LGG_00023	Metal-dependent membrane protease
LGG_00024	LGG_00024	Putative protein without homology
LGG_00026	is3	Transposase, IS5 family protein
LGG_00046	LGG_00046	Putative protein without homology
LGG_00074	LGG_00074	Conserved protein
LGG_00079	LGG_00079	Conserved protein
LGG_00080	zntR	Transcriptional regulator, MerR family
LGG_00081	LGG_00081	Conserved protein
LGG_00086	LGG_00086	Transcriptional regulator, TetR family
LGG_00087	LGG_00087	Conserved protein
LGG_00090	LGG_00090	PTS system, IICB component
LGG_00091	LGG_00091	Putative protein without homology
LGG_00092	frvA	PTS system, IIA component
LGG_00095	bglB	Beta-glucosidase (GH1)
LGG_00107	rmlC	dTDP-4-dehydrorhamnose 3,5-epimerase
LGG_00117	LGG_00117	Transcriptional regulator
LGG_00140	uvrB	Conserved protein
LGG_00141	LGG_00141	Putative protein without homology
LGG_00143	LGG_00143	Conserved protein
LGG_00144	is4	Transposase
LGG_00152	is5	Transposase
LGG_00153	is6	Transposase, IS4 family protein
LGG_00170	LGG_00170	Putative protein without homology
LGG_00171	LGG_00171	Conserved protein
LGG_00172	tauB	ABC transporter, taurine transporter ATP-b
LGG_00173	tauA	ABC transporter, aliphatic sulfonates fami
LGG_00174	tauC	Taurine transport system permease protein
LGG_00175	naoX	Pyridine nucleotide-disulphide oxidoreduct
LGG_00176	LGG_00176	Conserved membrane protein
LGG_00177	LGG_00177	Transcriptional regulator, LysR family
LGG_00209	LGG_00209	ABC transporter, ATPase and permease compo
LGG_00210	rrg	Transcriptional regulator, xre family
LGG_00235	is7	Transposase
LGG_00236	is8	Transposase, IS4 family protein
LGG_00278	LGG_00278	Conserved protein
LGG_00279	welA	dTDP-rhamnosyl transferase rfbF
LGG_00280	welB	alpha-L-Rha alpha-1,3-L-rhamnosyltransfera
LGG_00281	welC	alpha-L-Rha alpha-1,3-L-rhamnosyltransfera
LGG_00282	eps1	Polysaccharide Transporter, PST family pro

Gene Name	Symbol	Predicted Gene Product
LGG_00283	eps2	CpsH
LGG_00305	LGG_00305	Conserved protein
LGG_00306	LGG_00306	Conserved protein
LGG_03002	23S rRNA	23S ribosomal RNA
LGG_00307	LGG_00307	Conserved protein
LGG_00308	LGG_00308	Lipoprotein
LGG_00309	LGG_00309	Conserved protein
LGG_00329	upgB	ABC transporter, sugar transporter peripla
LGG_00330	LGG_00330	Putative protein without homology
LGG_00331	manA	Mannose-6-phosphate isomerase
LGG_00332	gntR	Transcriptional regulator, GntR family
LGG_00333	agaS	Tagatose-6-phosphate ketose/aldose isomera
LGG_00336	bgaC	Beta-galactosidase (GH35)
LGG_00338	manC	PTS system, mannose-specific IIC component
LGG_00339	manD	PTS system, mannose-specific IID component
LGG_00341	lacC	Tagatose-6-phosphate kinase
LGG_00342	srlD	Sorbitol-6-phosphate 2-dehydrogenase
LGG_00343	gatC	PTS system, galactitol-specific IIC compon
LGG_00344	is9	Transposase, IS5 family protein
LGG_00345	gatA	PTS system, galactitol-specific IIA compon
LGG_00346	gatB	PTS system, galactitol-specific IIB compon
LGG_00347	LGG_00347	Conserved protein
LGG_00351	patB	Aminotransferase
LGG_00352	ypdF	Aminopeptidase YpdF
LGG_00354	celA	PTS system, lactose/cellobiose-specific II
LGG_00355	chbA	PTS system, lactose/cellobiose-specific II
LGG_00356	ypdE	Aminopeptidase
LGG_00357	LGG_00357	Transcription antiterminator
LGG_00358	pepT	Peptidase T
LGG_00359	oppA	ABC transporter, oligopeptide-binding prot
LGG_00360	yhbS	Acetyltransferase
LGG_00361	LGG_00361	Conserved protein
LGG_00363	LGG_00363	ABC transporter, cobalt transporter permea
LGG_00364	cbiO	ABC transporter, cobalt transporter ATP-bi
LGG_00365	cbiQ	ABC transporter, cobalt transporter permea
LGG_00366	tenA	Transcriptional activator TenA
LGG_00367	thiW	Hydroxyethylthiazole permease
LGG_00369	thiE	Thiamine-phosphate pyrophosphorylase
LGG_00370	thiD	Phosphomethylpyrimidine kinase
LGG_00372	rbsR	Transcriptional regulator, LacI family
LGG_00373	rbsK	Ribokinase
LGG_00374	LGG_00374	Transcriptional regulator
LGG_00375	LGG_00375	Putative protein without homology

Gene Name	Symbol	Predicted Gene Product
LGG_00376	is10	Transposase, IS5 family protein
LGG_00377	LGG_00377	Putative protein without homology
LGG_00378	LGG_00378	Putative protein without homology
LGG_00379	LGG_00379	Putative protein without homology
LGG_00380	LGG_00380	Conserved protein
LGG_00381	LGG_00381	Conserved protein
LGG_00382	pbp	Penicillin-binding protein 3
LGG_00383	is11	Transposase, IS5 family protein
LGG_00384	LGG_00384	Putative protein without homology
LGG_00385	LGG_00385	Putative protein without homology
LGG_00386	LGG_00386	Transporter, major facilitator superfamily
LGG_00387	slyA	Transcriptional regulator, MarR family
LGG_00388	LGG_00388	Conserved protein
LGG_00389	LGG_00389	Putative protein without homology
LGG_00390	aroE	Conserved protein
LGG_00391	LGG_00391	Putative protein without homology
LGG_00392	LGG_00392	Putative protein without homology
LGG_00393	manR	Transcription antiterminator BglG family p
LGG_00394	LGG_00394	PTS system, IIA component
LGG_00395	frwA1	PTS system, fructose-specific IIA componen
LGG_00396	frwB1	PTS system, fructose-specific IIB componen
LGG_00397	frwC1	PTS system, fructose-specific IIC componen
LGG_00398	tal	Transaldolase
LGG_00399	rpe	Ribulose-phosphate 3-epimerase
LGG_00400	ulaA	PTS system, lactose/cellobiose-specific II
LGG_00401	ulaB	PTS system, lactose/cellobiose-specific II
LGG_00404	ulaC	PTS system, ascorbate-specific IIC compone
LGG_00405	tktN	Transketolase
LGG_00406	tktC	Transketolase
LGG_00407	scrK	Fructokinase
LGG_00408	lacR	Lactose phosphotransferase system represso
LGG_00409	frwA2	PTS system, fructose-specific IIA componen
LGG_00410	frwB2	PTS system, fructose-specific IIB componen
LGG_00411	frwC2	PTS system, fructose-specific IIC componen
LGG_00412	is13	Transposase, IS30 family protein
LGG_00413	fba	Fructose-bisphosphate aldolase
LGG_00414	gatY	Fructose-bisphosphate aldolase
LGG_00415	pts	PTS system, mannose-specific IIB component
LGG_00416	pts	PTS system, mannose-specific IIA component
LGG_00417	manZ	PTS system, mannose-specific IICD componen
LGG_00418	tal	Transaldolase
LGG_00419	LGG_00419	Transcriptional regulator, LacI family
LGG_00420	yhfZ	Transcriptional regulator, GntR family

Gene Name	Symbol	Predicted Gene Product
LGG_00421	LGG_00421	Conserved protein
LGG_00422	LGG_00422	Conserved protein
LGG_00423	LGG_00423	Conserved membrane protein
LGG_00424	php	Hydrolase
LGG_00425	yhfS	Pyridoxal phosphate-dependent transferase
LGG_00426	yhfX	Amino acid racemase
LGG_00427	YHFW	Phosphopentomutase
LGG_00428	ilvH	DNA-directed RNA polymerase subunit delta
LGG_00429	LGG_00429	Cobalt transport protein cbiQ
LGG_00430	cbiO	ABC transporter, cobalt transporter ATP-b
LGG_00431	cbiQ	Cobalt transport protein cbiQ
LGG_00432	LGG_00432	Putative protein without homology
LGG_00433	menC	N-acylamino acid racemase
LGG_00434	nagZ	Beta-N-acetylhexosaminidase (GH3)
LGG_00435	is14	Transposase, IS30 family protein
LGG_00436	tnpR	Resolvase
LGG_00437	LGG_00437	Conserved cytosolic protein
LGG_00438	LGG_00438	Putative protein without homology
LGG_00439	LGG_00439	Putative protein without homology
LGG_00440	LGG_00440	Putative protein without homology
LGG_00441	srtC1	Sortase family protein
LGG_00442	spaA	Pilus specific protein, major backbone pro
LGG_00443	spaB	Pilus specific protein, minor backbone pro
LGG_00444	spaC	Pilus specific protein, ancillary protein
LGG_00445	is15	Transposase, IS30 family protein
LGG_00446	LGG_00446	Conserved protein
LGG_00447	LGG_00447	Conserved protein
LGG_00448	LGG_00448	Putative protein without homology
LGG_00449	LGG_00449	UvrD/REP helicase
LGG_00450	ybjD	Putative protein without homology
LGG_00451	is16	Transposase, IS5 family protein
LGG_00452	is17	Transposase, IS3/IS911 family protein
LGG_00453	is18	Transposase, IS150/IS3 family protein
LGG_00454	is19	Transposase, IS150/IS3 family protein
LGG_00455	LGG_00455	Conserved protein
LGG_00456	pacL	Cation-transporting ATPase
LGG_00457	is20	Transposase
LGG_00458	is21	Transposase, IS4 family protein
LGG_00459	napA	Na/H antiporter
LGG_00462	is24	Transposase
LGG_00463	eriC	Chloride channel protein
LGG_00464	is25	Transposase, IS30 family protein
LGG_00465	LGG_00465	Transporter, major facilitator superfamily

Gene Name	Symbol	Predicted Gene Product
LGG_00466	is26	Transposase, IS5 family protein
LGG_00467	LGG_00467	Peptidase M20
LGG_00468	LGG_00468	Transcriptional regulator, LysR family
LGG_00469	LGG_00469	Putative protein without homology
LGG_00470	ebgA	Beta-galactosidase (GH42)
LGG_00471	ygjI	Amino acid permease family protein
LGG_00472	lysP	Lysine-specific permease
LGG_00473	araC	Transcriptional regulator, AraC family
LGG_00474	LGG_00474	Putative protein without homology
LGG_00475	LGG_00475	Transporter, major facilitator superfamily
LGG_00476	abgB	Aminobenzoyl-glutamate utilization protein
LGG_00477	LGG_00477	Opine/octopine dehydrogenase
LGG_00478	yecC	ABC transporter, amino acid transporter AT
LGG_00479	LGG_00479	ABC transporter, amino acid binding protei
LGG_00480	LGG_00480	ABC transporter, amino acid transporter pe
LGG_00481	LGG_00481	ABC transporter, amino acid transporter pe
LGG_00485	LGG_00485	Conserved protein
LGG_00487	LGG_00487	Putative protein without homology
LGG_00499	is27	Transposase, IS5 family protein
LGG_00500	metQ	ABC transporter, metal ion transporter per
LGG_00511	LGG_00511	ABC transporter, ATP-binding protein
LGG_00512	LGG_00512	ABC transporter
LGG_00513	is28	Transposase, IS5 family protein
LGG_00514	LGG_00514	Putative protein without homology
LGG_00515	LGG_00515	Putative protein without homology
LGG_00516	LGG_00516	Transcriptional activator
LGG_00517	is29	Transposase, IS5 family protein
LGG_00529	LGG_00529	Conserved protein
LGG_00533	radC	Conserved protein
LGG_00535	LGG_00535	Conserved protein
LGG_00559	LGG_00559	Transcriptional regulator, LysR family
LGG_00560	LGG_00560	Conserved protein
LGG_00561	LGG_00561	Sugar phosphate isomerase/epimerase
LGG_00562	kduD	Short-chain dehydrogenase/reductase SDR
LGG_00563	LGG_00563	Putative protein without homology
LGG_00564	aroE	Shikimate 5-dehydrogenase
LGG_00565	yfkL	Transporter, major facilitator superfamily
LGG_00566	aroE	Shikimate 5-dehydrogenase
LGG_00579	LGG_00579	Conserved extracellular protein
LGG_00580	LGG_00580	Putative protein without homology
LGG_00581	is30	Transposase
LGG_00582	is31	Transposase, IS4 family protein
LGG_00583	LGG_00583	Conserved protein

Gene Name	Symbol	Predicted Gene Product
LGG_00584	LGG_00584	Conserved protein
LGG_00585	LGG_00585	Conserved protein
LGG_00586	LGG_00586	Putative protein without homology
LGG_00589	LGG_00589	Conserved protein
LGG_00593	is32	Transposase, IS5 family protein
LGG_00594	LGG_00594	Putative protein without homology
LGG_00628	walR	Two-component response regulator
LGG_00629	walK	Two component sensor transduction histidin
LGG_00630	ycbN	ABC transporter, bacitracin transporter AT
LGG_00631	LGG_00631	ABC transporter, bacitracin transporter pe
LGG_00646	LGG_00646	Putative protein without homology
LGG_00647	LGG_00647	Putative protein without homology
LGG_00658	LGG_00658	Putative protein without homology
LGG_00659	pts	PTS system, galactose-specific IC compone
LGG_00660	LGG_00660	Putative protein without homology
LGG_00661	LGG_00661	Conserved protein
LGG_00662	LGG_00662	Beta-lactamase class C related penicillin
LGG_00663	LGG_00663	Conserved protein
LGG_00664	lacC	Tagatose-6-phosphate kinase
LGG_00703	LGG_00703	Regulator of polyketide synthase expressio
LGG_00704	LGG_00704	Short-chain dehydrogenase/reductase SDR
LGG_00705	adc	Acetoacetate decarboxylase
LGG_00813	LGG_00813	Conserved protein
LGG_00814	LGG_00814	Conserved protein
LGG_00815	LGG_00815	Conserved protein
LGG_00816	LGG_00816	Conserved protein
LGG_00817	LGG_00817	Conserved protein
LGG_03005	23S rRNA	23S ribosomal RNA
LGG_00818	LGG_00818	Conserved protein
LGG_00819	LGG_00819	Lipoprotein
LGG_00820	LGG_00820	Conserved protein
LGG_00912	is33	Transposase, IS150/IS3 family protein
LGG_00913	is34	Transposase, IS3 family protein
LGG_00950	LGG_00950	Conserved protein
LGG_00965	LGG_00965	Conserved protein
LGG_00973	LGG_00973	Putative protein without homology
LGG_00974	LGG_00974	Conserved protein
LGG_01023	LGG_01023	Adenine specific DNA methylase Mod
LGG_01024	is35	Transposase
LGG_01025	is36	Transposase, IS4 family protein
LGG_01026	LGG_01026	Type III restriction-modification system m
LGG_01027	res	Type III restriction-modification system r
LGG_01028	LGG_01028	Putative protein without homology

Gene Name	Symbol	Predicted Gene Product
LGG_01029	is37	Transposase, IS5 family protein
LGG_01063	LGG_01063	Conserved protein
LGG_01064	bglA	6-phospho-beta-glucosidase (GH1)
LGG_01086	int	Phage-related integrase
LGG_01087	LGG_01087	Putative protein without homology
LGG_01088	LGG_01088	Putative protein without homology
LGG_01089	LGG_01089	Conserved protein
LGG_01090	LGG_01090	Conserved protein
LGG_01091	LGG_01091	Putative protein without homology
LGG_01092	LGG_01092	Putative protein without homology
LGG_01093	LGG_01093	Putative protein without homology
LGG_01094	LGG_01094	Conserved protein
LGG_01095	Cpg	Phage-related transcriptional regulator, C
LGG_01096	LGG_01096	Putative protein without homology
LGG_01097	LGG_01097	Putative protein without homology
LGG_01098	LGG_01098	Putative protein without homology
LGG_01099	LGG_01099	Conserved protein
LGG_01100	LGG_01100	Putative protein without homology
LGG_01101	LGG_01101	Conserved protein
LGG_01102	LGG_01102	Conserved protein
LGG_01103	LGG_01103	Putative protein without homology
LGG_01104	LGG_01104	RecT family protein
LGG_01105	LGG_01105	Phage-related protein
LGG_01106	LGG_01106	Phage-related replication protein
LGG_01107	ssb3	Single-stranded DNA-binding protein
LGG_01108	LGG_01108	Phage-related protein with HTH-domain
LGG_01109	LGG_01109	Phage-related protein DNA-binding protein
LGG_01110	LGG_01110	Phage-related protein
LGG_01111	LGG_01111	Phage-related protein
LGG_01112	LGG_01112	Phage-related holliday junction resolvase
LGG_01113	LGG_01113	Phage-related protein
LGG_01114	LGG_01114	Phage-related protein
LGG_01115	LGG_01115	Phage-related protein
LGG_01116	LGG_01116	Putative protein without homology
LGG_01117	LGG_01117	Phage-related protein
LGG_01118	LGG_01118	Putative protein without homology
LGG_01119	LGG_01119	Putative protein without homology
LGG_01120	LGG_01120	Putative protein without homology
LGG_01121	LGG_01121	Phage-related protein
LGG_01122	LGG_01122	Phage-related HNH endonuclease
LGG_01123	LGG_01123	Phage-related protein, ribonucleoside-diph
LGG_01124	Rorf172	Phage-related terminase, small subunit
LGG_01125	Rorf447	Phage-related terminase, large subunit

Gene Name	Symbol	Predicted Gene Product
LGG_01126	LGG_01126	Phage-related portal protein
LGG_01127	LGG_01127	Phage-related Mu protein F like protein
LGG_01128	LGG_01128	Phage-related protein
LGG_01129	gpG	Phage-related minor capsid protein (GpG pr
LGG_01130	LGG_01130	Phage-related protein
LGG_01131	LGG_01131	Phage-related protein
LGG_01132	LGG_01132	Phage-related head tail joining protein
LGG_01133	LGG_01133	Phage-related major structural protein
LGG_01134	LGG_01134	Phage-related major tail protein
LGG_01135	LGG_01135	Phage-related protein
LGG_01136	LGG_01136	Phage-related protein
LGG_01137	LGG_01137	Putative protein without homology
LGG_01138	LGG_01138	Phage-related tail component
LGG_01139	LGG_01139	Phage-related tail-host interaction protei
LGG_01140	LGG_01140	Phage-related protein
LGG_01141	LGG_01141	Phage-related protein
LGG_01142	hol	Phage-related holin
LGG_01143	lys	Phage-related lysin (GH25)
LGG_01150	LGG_01150	Putative protein without homology
LGG_01151	LGG_01151	Putative protein without homology
LGG_01152	LGG_01152	Putative protein without homology
LGG_01154	LGG_01154	Putative protein without homology
LGG_01186	LGG_01186	Putative protein without homology
LGG_01212	LGG_01212	Conserved protein
LGG_01236	LGG_01236	Conserved protein
LGG_01243	LGG_01243	Chromosome segregation ATPase
LGG_01244	LGG_01244	Putative protein without homology
LGG_01246	LGG_01246	Putative protein without homology
LGG_01247	LGG_01247	Putative protein without homology
LGG_01248	is39	Transposase, IS5 family protein
LGG_01250	LGG_01250	Conserved protein
LGG_01253	LGG_01253	Transcriptional regulator, Xre family prot
LGG_01254	LGG_01254	DNA helicases
LGG_01316	LGG_01316	Putative protein without homology
LGG_01406	LGG_01406	Conserved protein
LGG_01515	LGG_01515	Integrase
LGG_01516	LGG_01516	Transcriptional regulator, Xre family
LGG_01517	LGG_01517	Conserved protein
LGG_01518	LGG_01518	Putative protein without homology
LGG_01519	LGG_01519	Phage-related endolysin
LGG_01520	LGG_01520	Phage-related holin
LGG_01521	LGG_01521	Phage-related holin
LGG_01522	LGG_01522	Phage-related infection protein

Gene Name	Symbol	Predicted Gene Product
LGG_01523	LGG_01523	Phage-related tail-host specificity protei
LGG_01524	LGG_01524	Phage-related tail component
LGG_01525	LGG_01525	Phage-related minor tail protein
LGG_01526	LGG_01526	Phage-related protein without homology
LGG_01527	LGG_01527	Phage-related tail component
LGG_01528	LGG_01528	Phage-related major tail protein
LGG_01529	LGG_01529	Phage-related tail component
LGG_01530	LGG_01530	Phage-related head-tail joining protein
LGG_01531	LGG_01531	Phage-related infection protein
LGG_01532	LGG_01532	Phage-related protein without homology
LGG_01533	LGG_01533	Phage-related prohead protease
LGG_01534	LGG_01534	Phage-related portal protein
LGG_01535	LGG_01535	Phage-related terminase large subunit
LGG_01536	LGG_01536	Phage-related terminase small subunit
LGG_01537	tnpR	Phage-related Resolvase
LGG_01538	LGG_01538	Phage-related glycosyl transferase, group
LGG_01539	is40	Transposase, IS5 family protein
LGG_01540	LGG_01540	Phage-related HNH nuclease
LGG_01541	ssb4	Single-stranded DNA-binding protein
LGG_01542	LGG_01542	Putative protein without homology
LGG_01543	LGG_01543	Putative protein without homology
LGG_01544	LGG_01544	Putative protein without homology
LGG_01545	rimL	Acetyltransferase, GNAT family protein
LGG_01546	LGG_01546	Conserved protein
LGG_01547	LGG_01547	ABC transporter, ATP-binding protein
LGG_01563	LGG_01563	ABC transporter, permease component
LGG_01564	mppX	ABC transporter, ATP-binding protein
LGG_01579	LGG_01579	NADPH-quinone reductase (Modulator of drug
LGG_01580	LGG_01580	Transcriptional regulator, TetR family
LGG_01581	is41	Transposase, IS5 family protein
LGG_01582	LGG_01582	Oxidoreductase
LGG_01583	LGG_01583	Putative protein without homology
LGG_01584	is42	Transposase
LGG_01585	is43	Transposase, IS4 family protein
LGG_01586	yohH	Glycosyl transferase, group 1
LGG_01587	yohJ	Glycosyl transferase, group 1
LGG_01588	LGG_01588	Putative protein without homology
LGG_01589	LGG_01589	Cell surface protein
LGG_01590	LGG_01590	Conserved protein
LGG_01591	LGG_01591	Conserved membrane protein
LGG_01592	LGG_01592	Putative protein without homology
LGG_01593	LGG_01593	Conserved protein
LGG_01622	is44	Transposase, IS3/IS911 family protein

Gene Name	Symbol	Predicted Gene Product
LGG_01623	is45	Transposase, IS150/IS3 family protein
LGG_01653	oppC	ABC transporter, oligopeptide transporter
LGG_01707	LGG_01707	Conserved protein
LGG_01728	LGG_01728	Endopeptidase M23B
LGG_01729	is46	Transposase, IS605 family protein
LGG_01730	LGG_01730	Putative protein without homology
LGG_01748	LGG_01748	Transcriptional regulator, Rrf2 family
LGG_01749	is47	Transposase, IS5 family protein
LGG_01750	LGG_01750	Transporter, major facilitator superfamily
LGG_01751	sir2	NAD-dependent deacetylase, SIR2-like prote
LGG_01755	LGG_01755	Conserved protein
LGG_01843	LGG_01843	Putative protein without homology
LGG_01848	LGG_01848	Conserved protein
LGG_01866	LGG_01866	Transcriptional antiterminator
LGG_01881	LGG_01881	Conserved protein
LGG_01886	LGG_01886	Conserved protein
LGG_01887	LGG_01887	Lipoprotein
LGG_01888	LGG_01888	Conserved protein
LGG_01890	LGG_01890	Conserved protein
LGG_01891	LGG_01891	Conserved protein
LGG_01892	LGG_01892	Conserved protein
LGG_01905	LGG_01905	Fic family protein
LGG_01927	LGG_01927	Conserved transmembrane protein
LGG_01928	LGG_01928	Putative protein without homology
LGG_01936	LGG_01936	Alpha/beta hydrolase superfamily protein
LGG_01937	gntR	Transcriptional regulator, GntR family
LGG_01938	LGG_01938	ABC transporter, ATPase component
LGG_01939	LGG_01939	ABC transporter, permease component
LGG_01940	oppF	ABC transporter, oligopeptide transporter
LGG_01945	oppA	ABC transporter, oligopeptide transporter
LGG_01950	LGG_01950	Type III restriction protein, res subunit
LGG_01951	LGG_01951	Aminoglycoside phosphotransferase
LGG_01952	LGG_01952	Zn-dependent endopeptidase, M10 family
LGG_01953	LGG_01953	Conserved protein
LGG_01954	lciIC	Transcriptional regulator, xre family
LGG_01955	LGG_01955	Reverse transcriptase-like protein
LGG_01956	is48	Transposase, IS5 family protein
LGG_01957	is49	Transposase
LGG_01958	LGG_01958	Putative protein without homology
LGG_01959	is50	Transposase, IS4 family protein
LGG_01960	LGG_01960	Conserved protein
LGG_01961	is51	Transposase, IS66 family protein
LGG_01962	LGG_01962	Transposase

Gene Name	Symbol	Predicted Gene Product
LGG_01963	LGG_01963	Conserved protein
LGG_01964	LGG_01964	Conserved protein
LGG_01965	is52	Transposase, IS5 family protein
LGG_01966	LGG_01966	Putative protein without homology
LGG_01967	LGG_01967	Conserved protein
LGG_01990	LGG_01990	Xylanase/chitin deacetylase
LGG_01991	LGG_01991	UDP-N-acetylglucosamine 2-epimerase
LGG_01992	LGG_01992	UDP-N-acetylglucosamine 2-epimerase
LGG_01993	LGG_01993	Conserved protein
LGG_01994	LGG_01994	Conserved protein
LGG_01995	LGG_01995	Conserved protein
LGG_01996	rmlD	dTDP-4-dehydrorhamnose reductase
LGG_01997	rmlB	dTDP-glucose 4,6-dehydratase
LGG_01998	rmlC	dTDP-4-dehydrorhamnose 3,5-epimerase
LGG_01999	rmlA	Glucose-1-phosphate thymidylyltransferase
LGG_02000	LGG_02000	Lyzozyme M1 (1,4-beta-N-acetylmuramidase)
LGG_02001	LGG_02001	Lyzozyme M1 (1,4-beta-N-acetylmuramidase)
LGG_02002	LGG_02002	Conserved protein
LGG_02003	is53	Transposase, IS5 family protein
LGG_02004	eps3	UDP-galactosephosphotransferase
LGG_02033	is54	Transposase, IS5 family protein
LGG_02038	rmlB	dTDP-glucose 4,6-dehydratase
LGG_02039	rmlC	dTDP-4-dehydrorhamnose 3,5-epimerase
LGG_02040	rmlA1	Glucose-1-phosphate thymidyl transferase 1
LGG_02041	is55	Transposase, IS5 family protein
LGG_02042	rmlA2	Glucose-1-phosphate thymidylyltransferase
LGG_02043	welE	Undecaprenyl-phosphate beta-glucosephospho
LGG_02044	welF	Glycosyl transferase, group 1
LGG_02045	welG	Glycosyl transferase,galactofuranosyltrans
LGG_02046	welH	alpha-L-Rha alpha-1,3-L-rhamnosyltransfera
LGG_02047	WelI	Glycosyl transferase, group 1
LGG_02048	welJ	Glycosyl transferase, alpha-1,3-galactosyl
LGG_02049	wzx	Polysaccharide Transporter, PST family pro
LGG_02050	glf	UDP-galactopyranose mutase
LGG_02051	LGG_02051	O antigen polymerase Wzy
LGG_02052	wze	Tyrosine-protein kinase capsular polysacc
LGG_02053	wzd	Chain length regulator (capsular polysacch
LGG_02055	LGG_02055	Phage-related infection protein
LGG_02056	LGG_02056	Phage-related infection protein
LGG_02062	oppF	ABC transporter, oligopeptide transporter
LGG_02063	oppD	ABC transporter, oligopeptide transporter
LGG_02066	oppA	ABC transporter, oligopeptide-binding prot
LGG_02087	LGG_02087	N-acetylmuramoyl-L-alanine amidase

Gene Name	Symbol	Predicted Gene Product
LGG_02092	LGG_02092	Conserved protein
LGG_02093	LGG_02093	ATP-dependent Lon protease
LGG_02094	LGG_02094	Conserved protein (PglZ domain)
LGG_02095	LGG_02095	Adenine-specific methyltransferase, Type I
LGG_02096	xerC	Phage-related integrase
LGG_02097	LGG_02097	Adenine-specific methyltransferase, Type I
LGG_02098	LGG_02098	Conserved protein
LGG_02099	LGG_02099	L-cystine import ATP-binding protein
LGG_02100	LGG_02100	Conserved protein
LGG_02160	is56	Transposase, IS4 family protein
LGG_02161	is57	Transposase
LGG_02165	is58	Transposase
LGG_02166	is59	Transposase, IS4 family protein
LGG_02171	is60	Transposase, IS5 family protein
LGG_02177	LGG_02177	Putative protein without homology
LGG_02178	yosT	Phage-related DNA gyrase inhibitory protei
LGG_02199	LGG_02199	Putative protein without homology
LGG_02200	LGG_02200	Putative protein without homology
LGG_02201	LGG_02201	CRISPR-associated protein, SAG0897 family
LGG_02202	cas2	CRISPR-associated protein, Cas2
LGG_02203	cas1	CRISPR-associated protein, Cas1
LGG_02204	csn1	CRISPR-associated protein, Csn1
LGG_02327	LGG_02327	Transcriptional regulator, Xre family
LGG_02336	LGG_02336	ABC transporter, multidrug transporter ATP
LGG_02358	LGG_02358	Conserved protein
LGG_02359	LGG_02359	Conserved protein
LGG_02373	LGG_02373	Conserved protein
LGG_02376	LGG_02376	Transcriptional regulator, Xre family
LGG_02380	LGG_02380	Prebacteriocin
LGG_02387	hpk3	Two component sensor transduction histidin
LGG_02427	LGG_02427	Conserved protein
LGG_02445	is62	Transposase, IS150/IS3 family protein
LGG_02446	is63	Transposase, IS3/IS911 family protein
LGG_02511	LGG_02511	Conserved protein
LGG_02512	LGG_02512	Conserved protein
LGG_02610	LGG_02610	Conserved protein
LGG_02611	LGG_02611	Conserved protein
LGG_02612	LGG_02612	Putative protein without homology
LGG_02613	ABC-NBD	ABC transporter, ATP-binding protein
LGG_02614	LGG_02614	ABC transporter, ATP-binding protein
LGG_02651	LGG_02651	Transcriptional regulator, GntR family
LGG_02652	LGG_02652	Alpha-L-fucosidase (GH29)
LGG_02653	pts	PTS system, IIAB component

Gene Name	Symbol	Predicted Gene Product
LGG_02654	levF	PTS system, IIC component
LGG_02655	levG	PTS system, IID component
LGG_02656	ubiD	3-octaprenyl-4-hydroxybenzoate carboxy-lya
LGG_02657	ubiX	3-octaprenyl-4-hydroxybenzoate carboxy-lya
LGG_02662	yniG	Transporter, major facilitator superfamily
LGG_02663	LGG_02663	Conserved protein
LGG_02664	dgoD	Galactonate dehydratase
LGG_02665	gatC	PTS system, galactitol-specific IIC compon
LGG_02666	gatB	PTS system, galactitol-specific IIB compon
LGG_02667	gatA	PTS system, galactitol-specific IIA compon
LGG_02668	kdgA	2-dehydro-3-deoxyphosphogluconate aldolase
LGG_02669	LGG_02669	Transcription antiterminator, BglG family
LGG_02670	celC	PTS system, cellobiose-specific IIA compon
LGG_02671	celA	PTS system, cellobiose-specific IIB compon
LGG_02672	bglA	Beta-glucosidase (GH1)
LGG_02673	ypbG	Sugar kinase and transkriptional regulator
LGG_02674	ypdC	Conserved protein
LGG_02675	LGG_02675	Alpha-mannosidase (GH38)
LGG_02676	is64	Transposase, IS5 family protein
LGG_02677	LGG_02677	Alpha-mannosidase (GH38)
LGG_02678	LGG_02678	PTS system, cellobiose-specific IIC compon
LGG_02679	gntR	Transcriptional regulator, GntR family
LGG_02680	fcsR	Fucose operon repressor, DeoR family
LGG_02681	LGG_02681	Class II aldolase/adducin domain protein
LGG_02682	fucU	L-fucose isomerase/ RbsD or FucU transpor
LGG_02683	ywtG	Transporter, major facilitator superfamily
LGG_02684	fucK	Carbohydrate kinase, FGGY family
LGG_02685	fucI	L-fucose isomerase
LGG_02686	LGG_02686	Putative protein without homology
LGG_02687	rhaD	Rhamnulose-1-phosphate aldolase
LGG_02690	rhaB	Rhamnulokinase
LGG_02694	LGG_02694	Conserved protein
LGG_02697	is65	Transposase, IS150/IS3 family protein
LGG_02698	is66	Transposase, IS3/IS911 family protein
LGG_02700	LGG_02700	Phage-related protein
LGG_02703	LGG_02703	Conserved protein
LGG_02742	LGG_02742	Conserved protein
LGG_02743	xylB	Xylulokinase
LGG_02744	LGG_02744	Sorbitol dehydrogenase
LGG_02745	esuD	fructose-bisphosphate aldolase
LGG_02746	pts	PTS system, mannose/fructose/sorbose-speci
LGG_02747	ahaB	PTS system, mannose/fructose/sorbose-speci
LGG_02748	ahaA	PTS system, mannose/fructose/sorbose-speci

Gene Name	Symbol	Predicted Gene Product
LGG_02749	LGG_02749	PTS system, mannose/fructose/sorbose-speci
LGG_02750	is67	Transposase, IS5 family protein
LGG_02751	fbaA	Fructose-bisphosphate aldolase
LGG_02752	LGG_02752	Carbohydrate kinase, FGGY family
LGG_02753	gatC	PTS system, galactitol-specific IIC compon
LGG_02754	gatB	PTS system, galactitol-specific IIB compon
LGG_02755	gatA	PTS system, galactitol-specific IIA compon
LGG_02756	fba	Fructose-bisphosphate aldolase
LGG_02757	farR	Transcriptional regulator, GntR family
LGG_02780	LGG_02780	Conserved protein
LGG_02870	is68	Transposase, IS3/IS911 family protein
LGG_02871	is69	Transposase, IS150/IS3 family protein
LGG_02874	LGG_02874	Conserved protein
LGG_02876	LGG_02876	Malate dehydrogenase
LGG_02877	malP	Citrate carrier protein
LGG_02879	dcuR	Two-component response regulator
LGG_02885	xerC	Phage-related integrase
LGG_02886	LGG_02886	Transcriptional regulator
LGG_02887	LGG_02887	Putative protein without homology
LGG_02888	LGG_02888	Conserved protein
LGG_02889	LGG_02889	Conserved protein
LGG_02890	LGG_02890	Conserved protein
LGG_02891	LGG_02891	Conserved protein
LGG_02892	LGG_02892	Conserved protein
LGG_02893	LGG_02893	Phage-related protein, DNA replication
LGG_02894	LGG_02894	Phage-related virulence-associated protein
LGG_02895	LGG_02895	Phage-related protein
LGG_02896	sb56	Phage-related HNH endonuclease
LGG_02897	terS	Phage-related terminase small subunit
LGG_02898	terL	Phage-related terminase large subunit
LGG_02899	LGG_02899	Phage-related conserved protein
LGG_02900	LGG_02900	Phage-related portal protein
LGG_02901	LGG_02901	Phage-related prohead protease
LGG_02902	LGG_02902	Phage-related head-to-tail joining
LGG_02903	LGG_02903	Putative protein without homology
LGG_02904	LGG_02904	Conserved extracellular protein
LGG_02905	ytgB	Transglycosylase-associated protein
LGG_02930	LGG_02930	Conserved protein
LGG_02944	tnp	Integrase

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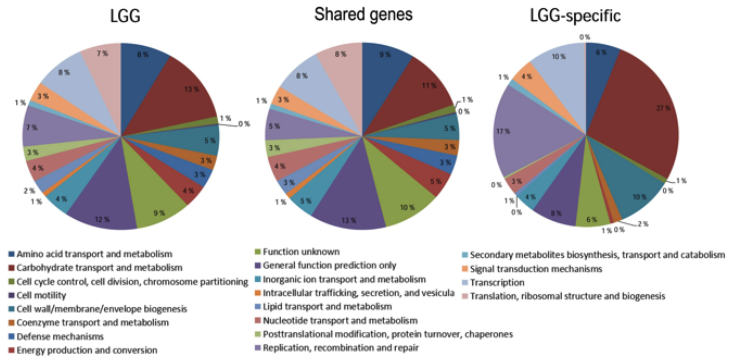
Additional Table 3: omitted

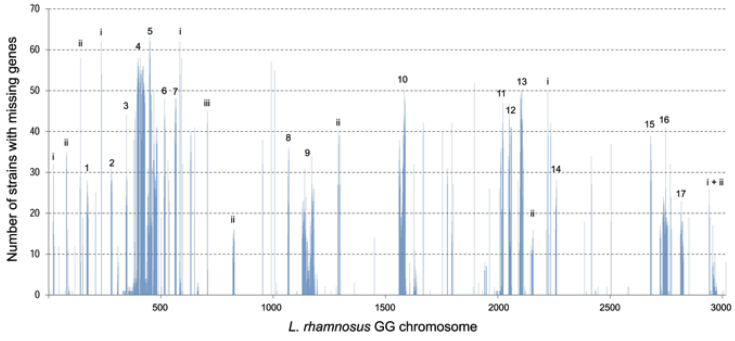
Additional Table S4

Bacteriophage hits											
Spacer	ID number	Organism/bactriophage/plasmid	Identities %	Hit Length	Mismatches	gaps	start	end	E-value	Bits	Host
spacer 4	gi|77696193|gb|AY131267.2|	Bacteriophage Lc-Nu, complete genome	92,86%	28	2	0	2157006	2159733	0,007	39,6	*Lactobacillus rhamnosus Lc1/3*
spacer 4	gi|166200914|gb|EU246945.1|	Lactobacillus phage Lrm1, complete sequence	89,29%	28	3	0	25407	25434	0,063	36,5	*Lactobacillus rhamnosus M1*
spacer 4	gi|22217797|emb|AJ251789.2|	Lactobacillus casei bacteriophage A2 complete genome	89,29%	28	3	0	24537	24564	0,063	36,5	*Lactobacillus casei ATCC 393*
spacer 6	gi|77696193|gb|AY131267.2|	Bacteriophage Lc-Nu, complete genome	90%	30	3	0	28562	28591	0,007	39,6	*Lactobacillus rhamnosus Lc1/3*
spacer 9	gi|22217797|emb|AJ251789.2|	Lactobacillus casei bacteriophage A2	92,86%	28	2	0	41435	41408	0,007	39,6	*Lactobacillus casei ATCC 393*
spacer 11	gi|57636010|gb|CP000029.1|	Staphylococcus epidermidis RP62A	89,66%	29	3	0	335894	335866	0,021	38	*Staphylococcus epidermidis RP62A*
spacer 12	gi|89953823|gb|DQ411856.1|	Lactobacillus casei phage Lca1	100%	28	0	0	36224	36251	9,00E-05	46	*Lactobacillus casei Lca1 prophage*
spacer 12	gi|47607149|gb|AY605066.1|	Bacteriophage phiAT3,	96,67%	30	1	0	34242	34213	9,00E-05	46	*Lactobacillus casei*
spacer 12	gi|22217797|emb|AJ251789.2|	Lactobacillus casei bacteriophage A2	100%	28	0	0	34348	34321	9,00E-05	46	*Lactobacillus casei ATCC 393*
spacer 18	gi|77696193|gb|AY131267.2|	Bacteriophage Lc-Nu	96,67%	30	1	0	34311	34340	9,00E-05	46	*Lactobacillus rhamnosus Lc1/3*
spacer 18	gi|47607149|gb|AY605066.1|	Bacteriophage phiAT3	96,67%	30	1	0	37765	37794	9,00E-05	46	*Lactobacillus casei*
spacer 21	gi|166200914|gb|EU246945.1|	Lactobacillus phage Lrm1, complete sequence	96,67%	30	1	0	7314	7343	9,00E-05	46	*Lactobacillus rhamnosus M1*
spacer 21	gi|22217797|emb|AJ251789.2|	Lactobacillus casei bacteriophage A2	96,67%	30	1	0	7526	7555	9,00E-05	46	*Lactobacillus casei ATCC 393*
spacer 22	gi|166200914|gb|EU246945.1|	Lactobacillus phage Lrm1,	100%	30	0	0	296	267	1,00E-05	49,1	*Lactobacillus rhamnosus M1*
spacer 22	gi|687918|gb|S73384.1|	orf1, brf2 (cohesive (single-stranded ends)) [Bacteriophage PL-1, host: Lactobacillus casei, Genomic, 65310 nt]	96,67%	30	1	0	611	582	9,00E-05	46	*Lactobacillus casei*
spacer 22	gi|22217797|emb|AJ251789.2|	Lactobacillus casei bacteriophage A2 complete genome	96,67%	30	1	0	296	267	9,00E-05	46	*Lactobacillus casei ATCC 393*
spacer 24	gi|22217797|emb|AJ251789.2|	Lactobacillus casei bacteriophage A2 complete genome	100%	30	0	0	6024	6053	1,00E-05	49,1	*Lactobacillus casei ATCC 393*
Plasmid hits											
spacer 14	gi|15722253|emb|AJ304453.1|	plasmid pSB102	84%	25	4	0	8958	8934	0,043	28,5	
spacer 20	gi|152449|gb|K03313.1|RIATL	Integrated Ri plasmid Agropine (A. rhizogenes strain A4) complete TL-DNA and flanking plant (Convolvulus arvensis) DNA	88%	25	3	0	2987	2963	0,005	31,7	

Additional Fig S1



Additional Fig S2



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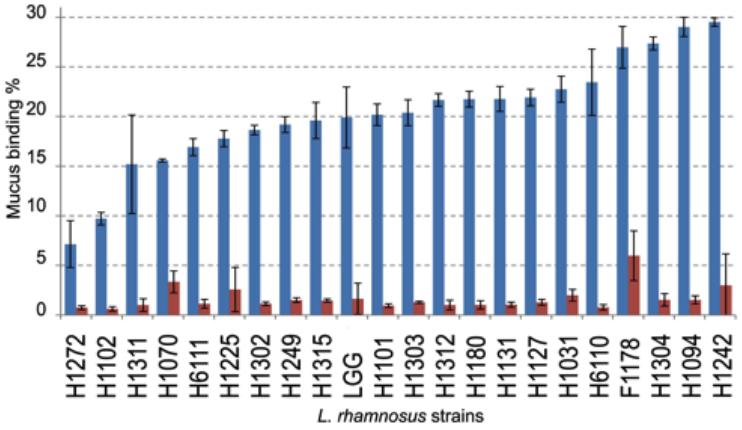
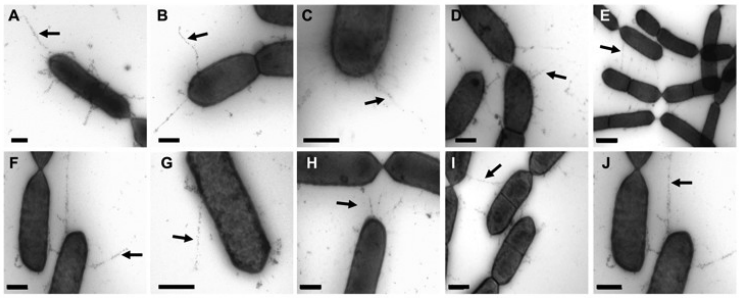
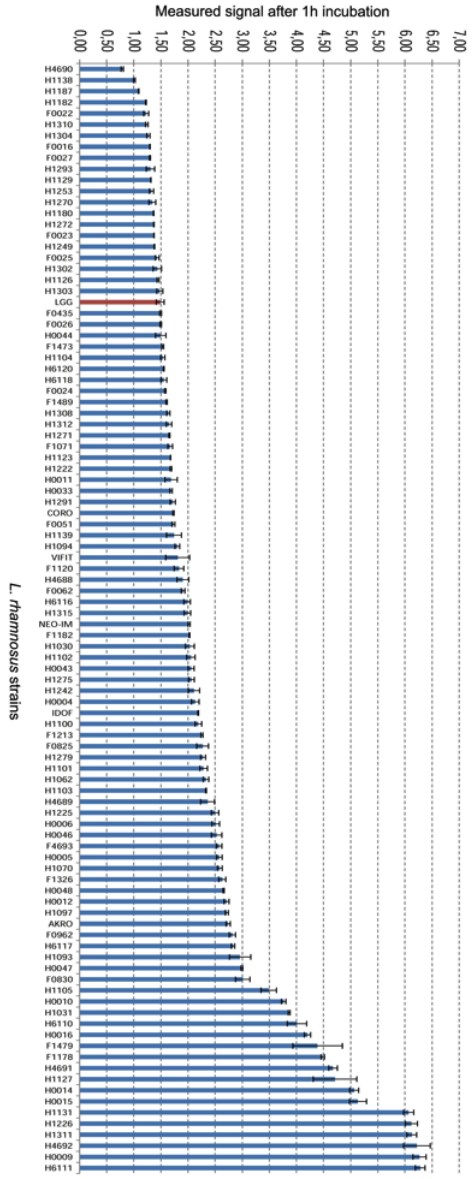
Additional Fig S3



Additional Fig S4



1312 **Additional Fig S5**



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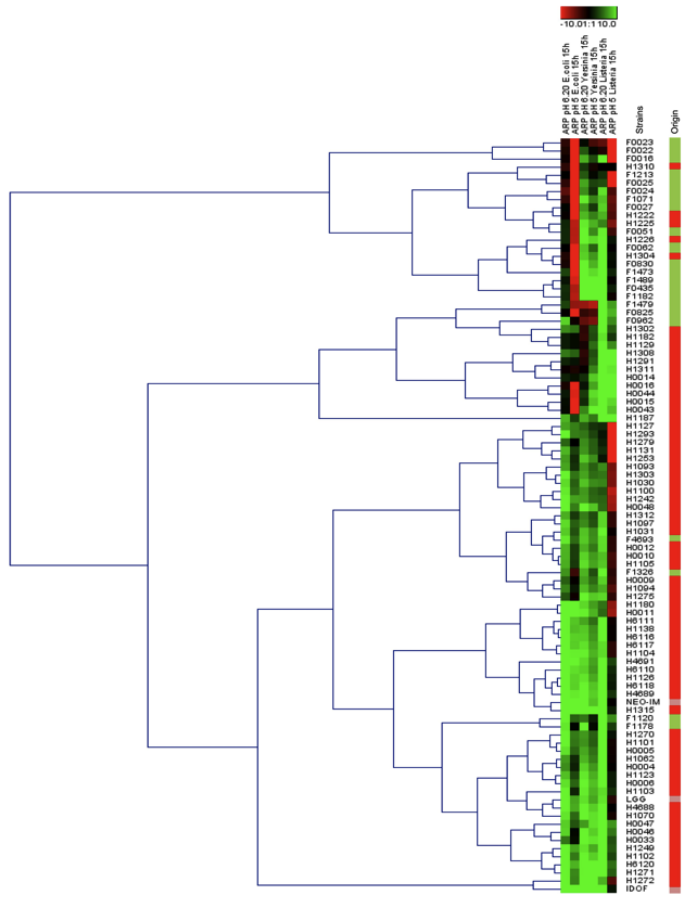
1314 **Additional Fig S6**



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