UNIVERSITÁ DEGLI STUDI DI CATANIA

Dipartimento di Agricoltura, Alimentazione e Ambiente

PhD Research in Food Production and Technology- XXVIII cycle

Alessandra Pino

Lactobacillus rhamnosus: a versatile probiotic species for foods and human applications

Doctoral thesis

Promoter

Prof. C.L. Randazzo

Co-promoter
Prof. C. Caggia

TRIENNIUM 2013-2015

Science knows no country, because knowledge

is the light that illuminated the word

TABLE OF CONTENTS

Chapter 1

General introduction and thesis outline

INTRODUCTION

The term *probiotic* is a relatively new word meaning "for life", used to designate microorganisms that are associated with the beneficial effects for humans and animals. These microorganisms contribute to intestinal microbial balance and play an important role in maintaining health. Several definitions of "*probiotic*" have been used over the years but the one derived by the Food and Agriculture Organization of the United Nations/World Health Organization (2001) (29), endorsed by the International Scientific Association for Probiotics and Prebiotics, best exemplifies the breadth and scope of probiotics. The latest are known today as "*live microorganisms which, when administered in adequate amounts, confer a health benefit on the host*". Following the FAO/WHO definition, the International Life Science Institute (ILSI) and the European Food and Feed Cultures Association (EFFCA) released similar definitions for probiotics: ''*a live microbial food ingredient that, when consumed in adequate amounts, confers health bene-fits on the consumers*'' and ''*live microorganisms that, when ingested or locally applied in sufficient numbers, provide the consumer with one or more proven health benefits*''.

Probiotics used in food, delivered as dietary supplement or as active components of a registered medication, should not only be able to survive passage through the digestive tract by exhibiting acid and bile survival, but should also have the capability to proliferate in the gastrointestinal tract (GIT). Probiotics must be able to exert their benefits on the host through growth and/or activity in the human body. Topical or local application of probiotics is also proposed in view of the recent evolution of scientific data (87). Therefore, the ability to remain viable and effective at the target site should be studied and confirmed for each strain, or even better, for each commercialized product.

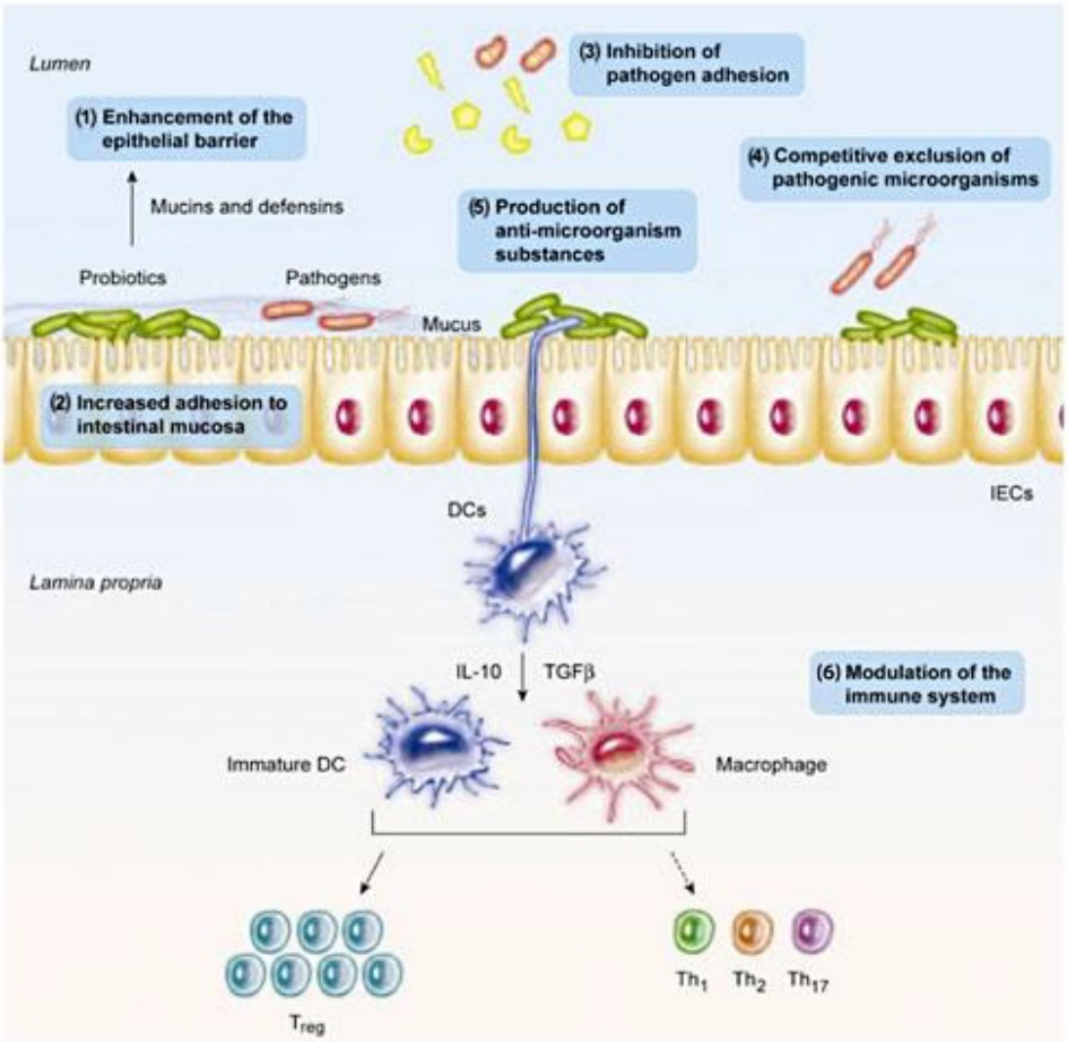
Moreover, clinical studies should be performed with the commercialized product and not with the isolated strain.

MECHANISM OF ACTION ON THE HOST

The probiotic potential of bacterial strains, even within the same species, is different. Indeed, various strains of the same species are always unique and may have different sites of adherence, specific immunological effects and actions. The mechanisms of action of probiotics include: receptor competition, mucin secretion and/or gut immune-modulation associated to lymphoid tissue, increase immunosuppressive and decrease pro-inflammatory mediators (39). Probiotic agents exert a beneficial effect via a wide array of actions (figure 1.1). These include resistance to colonization, production of antimicrobial substances, inhibition of pathogens adhesion, degradation of toxins, stimulation of local and peripheral immunity, stimulation of brush border enzyme activity, stimulation of secretory-IgA, prevention of microbial translocation (6, 120). The list of health benefit accredited to the probiotics continues to increase and up to now, several scientific studies have demonstrated therapeutic and preventive evidences. Regarding to the therapeutic applications it has been shown that probiotics are able to help the regulation of intestinal microbiota, to flaunt immunomodulatory properties, to reduce lactose intolerance symptoms and to enhance nutrients bioavailability. They also decrease the prevalence of allergies in susceptible individuals, inhibit the inflammatory responses in the gut and have antagonistic effects against intestinal and food borne pathogens. Probiotic strains typically colonize the intestinal tract then reinforce the host defence systems by inducing the mucosal immune responses. Reports indicate that some of this, particularly lactobacilli and bifidobacteria, are effective to enhance

the innate and the adaptive immunity, to prevent the gastric mucosal lesion development, to alleviate allergies and to put up defence against intestinal pathogen infections.

Figure 1.1 Probiotic modes of action.



Probiotics are assumed to exert their health-beneficial effects through different mechanisms of action. First of all, by pathogen exclusion through competition for adhesion places and nutrients and by the production of antimicrobial substances. Second, by enhancing the epithelial barrier function, for instance via the production of defensins and mucins. Finally, by modulation of host immune responses (34).

THE MAIN PROBIOTIC STRAINS

The most popular probiotic strains are represented by the genera *Lactobacillus* and *Bifidobacterium* but other organisms including enterococci and yeasts have also been used as probiotics. *Lactobacillus* is a bacterial genus comprised of Gram-positive, rod-shaped bacteria with a low percentage of guanine and cytosine bases in their genome, and they are typically aerotolerant anaerobes. Taxonomically, the *Lactobacillus* genus is diverse and it contains at least twelve different phylogenetic groups (26). More than 150 species have been named within the *Lactobacillus* genus, which were isolated, e.g., from human and animal gastrointestinal tracts (GITs) and mucous membranes and from plant surfaces. Several *Lactobacillus* strains are used in the preparation of fermented dairy products and in the production of sauerkraut, pickles, and silage. Certain *Lactobacillus* strains have been found to have beneficial effects on human health, some of which are, therefore, used as probiotics. One of the most important probiotic *Lactobacillus* strain is *L. rhamnosus* GG (LGG), which is probably the most intensively studied probiotic bacterium worldwide.

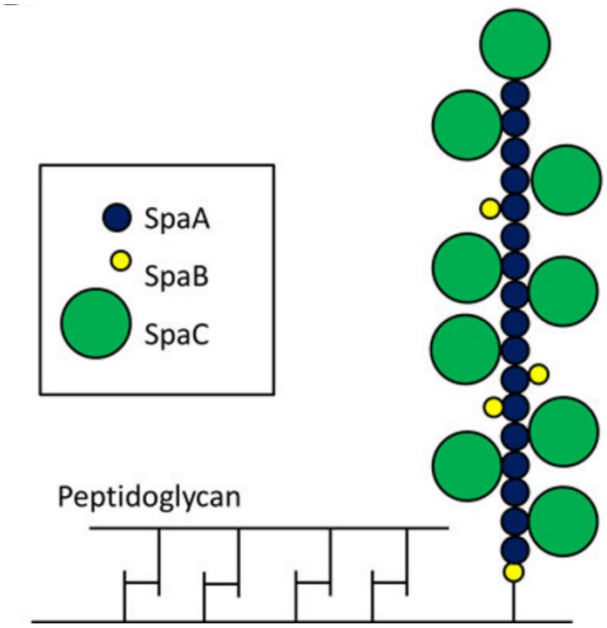
Lactobacillus rhamnosus GG

The probiotic strain *L. rhamnosus* GG (ATCC 53103) was originally isolated by Goldin and Gorbach in 1985 (20). Strain GG was selected from a collection of *Lactobacillus* strains isolated from stool samples from healthy human volunteers, using the criteria for an ideal probiotic strain (20). The first report of positive health effects of LGG was published in 1987 (38), and more than 500 scientific articles on this probiotic strain have since been published. The health effects of LGG are based on several mechanisms. LGG colonizes the GIT efficiently and

competes for adhesion sites and nutrients, such as monosaccharides, with pathogens. It also modulates the microecology of the GIT, e.g., by producing short-chain fatty acids, which favour the growth of non-pathogenic organisms. In addition, LGG has numerous effects on the host immune system (20). Recently, pili were identified on the cell surface of LGG. Studies have demonstrated that the main function of LGG pili is to mediate adherence to mucus glycoproteins and to human intestinal epithelial cells (49, 53, 21). In this case, adhesion does not result in infection but in a prolonged and stable persistence in the GIT, which is an important requirement to exert health benefits. Adhesion occurs through the zipper-like mechanism as in Gram-positive pathogens, described above (53). Besides the *SpaC* adhesin, also the *MabA* protein of LGG plays an important modulating role for the interaction with the host (114). Based on the genome sequence, it was predicted that LGG possesses two pilus gene clusters, *spaCBA* and *spaFED*, which are located distantly from each other as individual islands in the GG genome (49). Reunanen and co-workers (88) used Western blotting and immunogold transmission electron microscopy (TEM) in combination with antibodies against recombinant *SpaCBA* and *SpaFED* pilins to determine the presence of these two putative pili of LGG. Based on their findings, only *SpaCBA* pili were present. A possible explanation for the absence of *SpaFED* pili could be the lack of appropriate environmental stimuli to activate the associated gene cluster of *SpaFED*, so these pili might still be produced in the GIT. LGG cells contain multiple *SpaCBA* pili, on average 10-50 per cell with a length of up to 1 µm. These pili are heterotrimeric, composed by three subunits: *SpaA*, *SpaB* and *SpaC*, and all participate in the assembly of the pilus fibre (Figure 1.2). *SpaCBA* pili are assembled through the sortase system, as described for Gram-positive bacteria. *SpaA* is again the major subunit that builds up the pilus backbone, while *SpaB* and *SpaC* are the minor accessory subunits. In particular,

SpaC is the adhesin pili subunit responsible for the binding to human mucus and to Caco-2 intestinal epithelial cell line and it is located not only at the pilus tip but also along the length of the pilus shaft (53). *SpaB* is thought to have dualistic characteristics: (i) acting as a molecular switch that terminates pilus polymerization and predesignates mature pili to be covalently attached to the peptidoglycan by the housekeeping sortase and (ii) having a role as an adhesion allowing its binding to human intestinal mucus; this ability, however, cannot be compared to that of the SpaC subunit (118, 88).

Figure 1.2 Schematic presentation of the SpaCBA pili.



SpaCBA pili are composed of three different subunits. The SpaA subunit builds up the backbone of the pilus fibre, SpaC is an important adhesin, located at the tip and along the length of the fibre and SpaB is located at the base (figure adapted from Reunanen *et al.*, 2012 (88)).

The immunomodulatory capacity of LGG-pili was also studied. Lebeer and co-workers (53) compared the cytokine expression profiles in a Caco-2 cell line stimulated with LGG wild type and two mutants, a pilus mutant strain CMPG5357 (*spaCBA* mutant) and an EPS-deficient mutant CMPG5351 (*welE* mutant) with an enhanced exposure of the pili. Results showed an up regulation of pro-inflammatory cytokines such as interleukine-8 (IL-8) and tumor necrosis factor α (TNF-α) and a down regulation of anti-inflammatory IL-10 when cells were treated with the *spaCBA* mutant without pili. In contrast, an inverse tendency was observed when cells were stimulated with the *welE* mutant, indicating that pili of LGG might play a key role in immunomodulatory interactions with intestinal epithelial cells (IECs). Nevertheless, future studies are necessary to gain more insight in this pilus-mediated immunomodulatory activity. LGG is up to now one of the few probiotics that are known to produce mucus-binding pili, providing the ability to adhere to human IECs. Pilus-like structures were also reported in some different *Bifidobacterium* species as *Bifidobacterium bifidum*, *B. longum* subsp. *longum* and *B. animalis* subsp. *lactis* but the function of these structures still needs to be elucidated (30). In *B. breve* UCC2003, the presence of tight adherence (Tad) pili was demonstrated to be required for colonization and persistence of the strain in the murine gut (68). Furthermore, genes for sortase dependent pili were also discovered in *B. bifidum* PRL2010. Expression of the coding sequences in non piliated *L. lactis* increased the adherence of this strain to human enterocytes, indicating an important role for these pili in adhesion (113). Recently, a natural isolate of *L. lactis* (TIL448 strain) with high adhesion capacity to Caco-2 cells, compared to other *L. lactis* strains, was characterized. Proteomic analysis revealed the presence of pilins at the cell surface of *L. lactis* TIL448, that might be responsible for this increased adhesion capacity (62). The production of adherent pili might contribute to the competitive advantage

of LGG- and some other probiotic strains- over other species in the mucosal environment and also to exert their health benefit effects.

PROBIOTIC IN FOOD APPLICATIONS

Modern consumers are increasingly interested in their personal health, and expect the food that they eat to be healthy or even capable of preventing illness. The list of health benefits accredited to functional foods continues to increase and the probiotics are one of the fastest growing categories within food for which scientific researchers have demonstrated therapeutic evidences. In this context, probiotic cultures are increasingly being added to foods in order to develop products with health-promoting properties. The probiotic foods market has been very promising and it is expected to reach US$ 8.6 billions by 2015, representing an increase of 21% compared to 2009 (107). The majority of foods containing probiotics are currently dairy based and in particular yogurt and cheese. Probiotic yogurts are subjected to several regulatory requirements related in particular to the number of viable bacteria contained and pH value. In fact, it is crucial that probiotics are alive at the time of use and the viable colony forming unit (CFU) count must be at the level proven to confer a health benefit in humans or animals. Studies showed that probiotic microorganisms generally grow slowly in milk (69, 58, 17) and often the probiotic survival and viability in yogurts are lower than needed for the daily recommended intake (57). Low numbers of *Lactobacillus acidophilus* and especially bifidobacteria have been found in commercial yogurts (100). Among probiotic dairy foods, cheeses are advantageous as a delivery system for viable probiotics due to their intrinsic properties. Important is the pH value, higher than pH of fermented milks, that provides a more stable and favourable environment during ripening. Moreover, the high fat

content of cheeses protects the probiotic bacteria during their passage though the peptic system (77). The most difficult task in the production of probiotic cheese is not the growth of the probiotic bacteria but their survival. This is due to the fact that there is a long period of ripening, commonly several weeks or even months, in which the bacteria have not only to survive but also to survive in significant numbers to beneficially affect human health. There is a minimum concentration of microorganisms in these foods, which is variable. The most widely accepted concentration of probiotic microorganisms is 10^6 CFU per milliliter or gram, which is also set as industrial standard. Several types of cheeses are prepared by the incorporation of probiotic bacteria (Table 1.1) and up to now new products are being introduced in the international market, such as milk-based desserts, powdered milk for new-born infants, ice-creams, butter, mayonnaise, etc. (10, 51, 94).

Even if fermented dairy products generally provide good carrier matrices of probiotic microorganisms, the increasing number of individuals with lactose intolerance, dyslipidemia, and vegetarian or vegan diet reinforce the importance of development of non-dairy probiotic products such as fruit and vegetables (75, 81). Application of probiotic cultures in non-dairy products represents now a great challenge. Several studies reported that beverages such as fruit and vegetable juices may be the next category of food matrices to serve as carriers of probiotic bacteria, as supported by published studies (66, 67, 74, 104, 80, 46). Besides, probiotic foods containing fruit ingredients are increasingly preferred by consumers (24) and in addition to fruits, raw and fermented vegetables and cereals have been used as substrates for probiotic bacteria once these foods contain nutrients easily assimilated by probiotics (52, 75, 105). Strains of *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus* and *Bifidobacterium lactis* are the most utilized in the formulation of new vegetable probiotic

products. Products made from fruits and vegetables, such as drinks, purées, fermented vegetables, table olives (75) and minimally processed fruit (90, 91) have also been used. Thus, the development of non-dairy probiotic products, including food matrices from fruit, vegetables and cereals (Table 1.2), has a promising future. Besides, several studies have reported the use of probiotic bacteria with high amount of cells in fruit juices, fruit smoothies, minimally processed fruit, fermented vegetables, snack products, olive, and cereal beverage to provide probiotic food free of cholesterol, lactose and allergens present in dairy products (Table 1.3). However, future studies should be done to assess probiotic adherence and viability in the human intestine by means of in vivo clinical trials.

Table 1.1 Cheeses with incorporated probiotic bacteria.

Cheese type	Incorporated probiotic bacteria	References
Minas fresh cheese	*Lactobacillus acidophilus*	18
	L. acidophilus, S. thermophilus	106
	B. bifidum	31
Fresh cheese	*L. acidophilus*	60
Crescenza	*Bifidobacteria*	35
Cottage	*Bifidobacteria*	5, 92
Soft cheese	*Lattobacillus spp.*	14
Petit Suisse cheese	*B. animalis subsp. lactis, L. acidophilus*	7
	L. acidophilus, B. animalis	73
Argentinean fresco	*Bifidobacterium, L. acidophilus,L. casei*	116
	L. paracasei	117
Kariesh cheese	*Bifidobacterium*	65
Cremoso cheese	*L. casei, L. plantarum, L. rhamnosus*	63
Mascarpone cheese	*Streptococcus thermophilus*	8
Gouda	*L. acidophilus, B. lactis*	36
	L. paracasei	43
Pategràs cheese	*L. acidophilus, L. paracasei, B. lactis*	3
	L. casei, L. plantarum, L. rhamnosus	63
Probiotic goat's cheese	*Lactobacillus delbrueckii*	27
	L. acidophilus, B. lactis	37
Festivo cheese	*L. acidophilus, Bifidobacterium*	93
Canestrato Pugliese	*B. longum, B. bifidum*	16
Tallaga cheese	*L. acidophilus, B. B. lactis*	23
Cheddar	*L. acidophilus, Bifidobacterium sp., L. casei, L. paracasei, L. rhamnosus*	76
	L. casei	102
	L. paracasei	33
	B. lactis, B. longum	61
	B. longum, B. lactis,L. paracasei, L. acidophilus, L. casei	70, 71

Table 1.2 Recent studies to the use of vegetable food matrices for carrying probiotic bacteria.

Food matrix	Probiotic microorganism	Food product	Reference
Apple	*L. rhamnosus GG*	Minimally processed product	1, 90, 91
	L. casei		22
Cocoa	*B. longum*	Chocolate	79
	L. helveticus		
Cashew	*L. jonsonii*	Juice	115
Orange, grapefruit, black currant, pineapple, pomegranate, cranberry and lemon	*L. plantarum*	Juice	67
Yam	*L. acidophilus*	Fermented yam	55
Pomegranate	*L. plantarum, L. delbrueckii, L. acidophilus* and *L. paracasei*	Juice	64
Shalgam	*L. plantarum*	Drink	111
Pear	*L. acidophilus*	Juice	2
Plum	*L. kefiranofaciens, C. kefir, S. boluradii*	Juice	104
Soybean	*L. acidophilus*	Soybean bar	12
Barley, tomato pulp and whey powder	*L. acidophilus*	Food mixture	48
Cabbage	*L. plantarum*	Sauerkraut	123

Adapted from Furtado Martins et al., 2013 (32).

Table 1.3 Viability of probiotic cultures in fruit and vegetable products.

Food product	Probiotic microorganism	Viability of the probiotic in the product	Reference
Tomato juice	*L. acidophilus* LA39, *L. casei* A4, *L. delbrueckii* D7 and *L. plantarum* C3	10^6–10^8 CFU mL^{-1}	120
Beet juice	*L. acidophilus* LA39, *L. casei* A4, *L. delbrueckii* D7 and *L. plantarum* C3	10^6–10^8 CFU mL^{-1}, except for *L. acidophilus*	121
Cabbage juice	*L. casei* A4, *L. delbrueckii* D7 and L. *plantarum* C3	10^7 CFU mL^{-1} of *L. plantarum* and 10^5 CFU mL^{-1} of *L. delbrueckii*. *L. casei* did not survive	122
Fruit smoothies	*L. acidophilus* LA-5 and *B. animalis* ssp. *lactis* BB-12	10^7 CFU g^{-1}	89
Orange, pineapple and cranberry juice	*L. casei* DN 114 001, *L. rhamnosus* GG, *L. paracasei* NFBC 43338, and *B. lactis* Bb-12	Above 10^7 CFUmL^{-1} of *L. casei*, *L. rhamnosus* and *L. paracasei* in orange juice and above 10^6 CFU mL^{-1} in pineapple juice. *B. lactis* showed concentrations above 10^6 CFU mL–*1* in both juices	103
Olive	*L. paracasei* IMPC2.1	10^7 CFU g^{-1}	19
Cataloupe juice	*L. casei* NRRL B-442	Above 10^8 CFU mL^{-1}	28
Minimally processed apple and papaya	*B. lactis* BB-12	Above 10^6 CFU g^{-1}	112

Food product	Probiotic microorganism	Viability of the probiotic in the product	Reference
Fruit cocktail from carrot, celery and apple	*L. acidophilus* LA-5	Above 10^7 CFU mL^{-1}	66
Minimally processed symbiotic apples	*L. rhamnosus* GG	10^7–10^8 CFU g^{-1}	91
Honeydew melon juice	*L. casei* NCIMB 4114	Above 10^8 CFU mL^{-1}	99
Cashew juice	*L. casei* NRRL B 442	Above 10^8 CFU mL^{-1}	74
Turşu	*L. plantarum* BC 7321	Above 10^7 CFU mL^{-1}	9
Defatted soy flour	*L. paracasei* BCRC 14023 and *Bifidobacterium longum* BCRC 14661	Above 10^9 CFU g^{-1}	11
Snack product from apple, mandarin and pineapple grape juice	*L. salivarius* spp. *salivarius* CETC4063 and *L. acidophilus* CECT903	Above 10^7 CFU g^{-1}	4
Beverage from rice, barley, oats, wheat, soy flour and red grape juice	*L. plantarum* 6E and M6	Above 10^8 CFU g^{-1}	13
Malt beverage	*L. plantarum* NCIMB 8826 and *L. acidophilus* NCIMB 8821	Above 10^8 CFU mL^{-1}	82

Table adapted from Furtado Martins et al., 2013 (32)

PROBIOTICS IN HUMAN APPLICATION

Clinical trials conducted on infants, child and adult have demonstrated the usefulness of probiotic supplementation for treat gut, lung, skin, vaginal and overall diseases (figure 1.3). Beneficial effects of probiotic strains, turned out and proposed, include the following:

- improvement nutritional value of the food by increasing absorption of vitamins and minerals and better digestibility;
- promotion of intestinal lactose digestion by increasing tolerance of lactose due to microbial hydrolysis (56);
- positive influence on intestinal microbiota combating aberrant microbiota (47);
- positive influence on urogenital tract. In fact, in urinary tract infections probiotics are able to inhibit *E. coli* growth moreover, in bacterial vaginitis and vaginal candidosis the probiotic administration is capable to restore vaginal lactobacilli microbiota. Probiotic supplementation is able to inhibit the growth of pathogenic bacteria and *Candida spp.* (85, 86, 25);
- prevention and reduction of intestinal tract infections such as: acute gastroenteritis and antibiotic-associated diarrhea by reducing duration of infections (44, 42, 110); inflammatory bowel diseases by enhancing of mucosal immune responses, by improving of intestinal permeability, by lowering disease activity and by reducing relapse rates (101);
- regulation of gut motility associated particularly to constipation by shortening of intestinal transit time through modulation of colonic microbiota (40);

- improvement of immune system;
- prevention of colorectal cancer by down-regulation of intestinal microbial enzyme activities and by conversion of pre-carcinogens to carcinogens (15);
- alleviation of symptoms and reduction of risk of atopic eczema (83, 84);
- regulation of the inflammatory status in the Inflammatory Bowel Diseases (Crohn's disease and Ulcerative Colitis);

Other innovative benefits that are attributed to probiotics are:

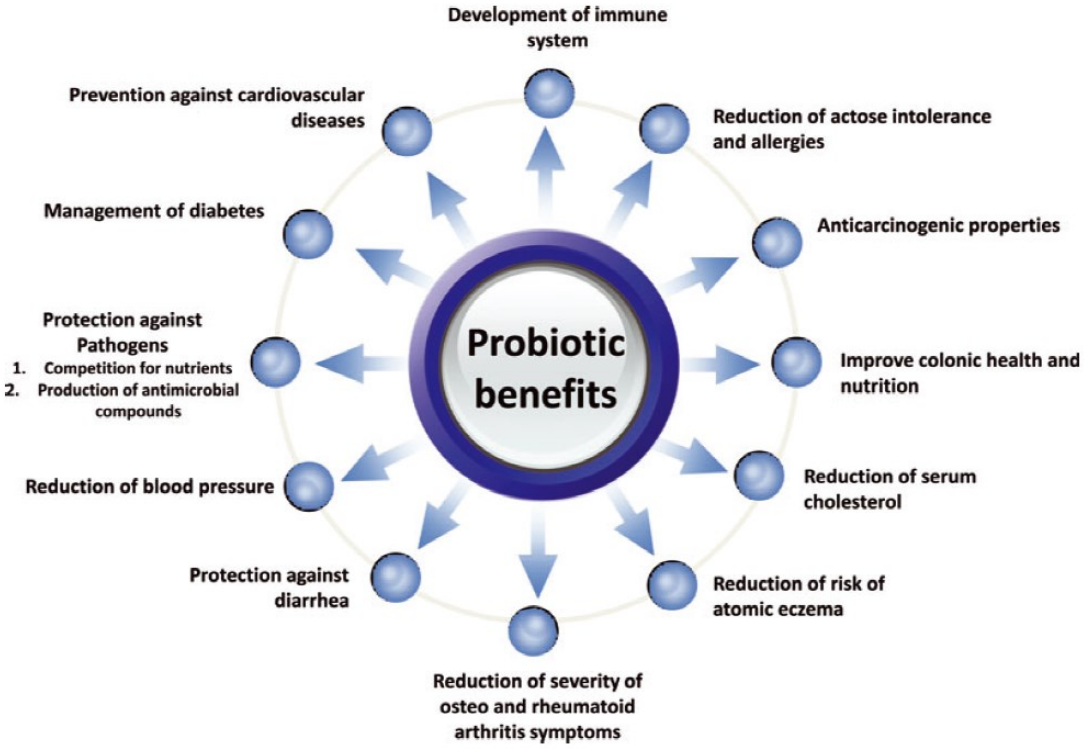
- hypocholesterolemic activity: mediated either by Bile Salts Hydrolases (BSH) enzymes secreted by some probiotics able to de-conjugate bile acids in the gut lumen or by direct cholesterol absorption (72). Both mechanisms of action decrease the plasmatic concentration of cholesterol, particularly beneficial in case of mild to severe hypercholesterolemia;
- inhibition of nitrate reduction: the organic acids produced by bifidobacteria and lactobacilli are able to inhibit the development of many intestinal nitrate reducing bacteria (e.g. *Escherichia coli, Proteus, Pseudomonas*) (50). The nitrite ions oxidize hemoglobin ferrous ions to ferric ions transforming it into methaemoglobin, typically unable to bind oxygen. This is a particular risk in infants, for which it is important to avoid taking water rich in nitrates (45);
- synthesis of group B vitamins: certain strains, especially belonging to *Bifidobacterium* genus and *Lactobacillus reuteri* species, have the capability to synthesize water-soluble

vitamins such as those included in the B group as folates (vitamin B9), biotin (vitamin B7), cobalamin (vitamin B12) and riboflavin (vitamin B2) (78, 108, 54);

- reduction of oxidative stress: specific microorganisms are able to produce antioxidant molecules, primarily glutathione and superoxide dismutase (SOD), which could be absorbed through the intestinal wall and exert a systemic positive impact on the average level of oxidative stress (41, 59);

- treatment of gaseous colic in infants: it is well-known that many new-borns and infants suffer from gaseous colic, often responsible for prolonged crying spells and severe discomfort. Differences in gut microbiota were found among colicky and non-colicky infants: colicky infants were less frequently colonized by *Lactobacillus* spp. and more frequently by anaerobic Gram-negative bacteria. Many of these, belonging to the genera *Enterobacter* and *Klebsiella*, have been isolated from colicky infants and they are thought to be directly involved in colic onset and maintenance. In fact, gas forming coliforms may be involved in determining colonic fermentation and consequently excessive intra-intestinal gas load, aerophagia, and pain, characteristic symptoms of colic crying. Only some very specific probiotic microorganisms, many of them isolated from infants, have the ability to directly inhibit such Gram-negative bacteria, thus offering a significant relief to colicky babies (96-98, 109).

Figure 1.3 Major benefits of probiotics for human nutrition and health.



Plessas et al. 2012 (77)

RESEARCH AIMS AND THESIS OUTLINE

The research described in this thesis aims to study the versatility of probiotic strains belonging to the *Lactobacillus rhamnosus* species in order to make renewed probiotic foods and to formulate clinical strategy to restore homeostasis condition in different human ecosystems. To these ends *L. rhamnosus* H25 strains, belonged to the D3A collection and previously isolated from Pecorino Crotonese cheese, was studied in order to evaluate functional foods feasibility. In addition, to study the usefulness of probiotic administration in human, the ability of *L. rhamnosus* GG strain to restore vaginal and intestinal ecosystems homeostasis was studied.

Actually, the demand for probiotic foods is increasing in Europe, Japan and the U.S. reflecting the heightened awareness among the public of the relationship between diet and health. Traditionally, the most popular food delivery system for probiotics have been freshly fermented dairy foods, such as yogurts, fermented milks and fresh cheeses. Most studies on probiotic strains are based on functional properties and less knowledge is available concerning their capacity to withstand stress related to food manufacturing and storage. Cheese as a probiotic food carrier represents a good choice showing potential advantages over other dairy fermented products but it is also a technological challenge at the same time. The technological suitability of probiotic strains poses a serious challenge since their survival and viability may be adversely affected by processing conditions as well as by the product environment, the storage conditions and the ripening time. To date few information are available concerning the ability of probiotic strains to survive during prolonged ripening time and no information is available about the possibility to use the Pecorino Siciliano cheese as carrier of probiotic strains. According to that, two promising probiotic strains, *Lactobacillus rhamnosus* H25 and

Lactobacillus paracasei N24, were used for the development of probiotic Pecorino Siciliano cheese (**chapter 2**) and their viability and influence on sensory properties, during the ripening time, were monitored. Moreover, the ability of H25 and N24 strains to survive throughout the gastro intestinal tract was also studied performing an *in vivo* test with 10 healthy volunteers. Recently, vegetarian fermented foods have reached an increasing attention as working base alternative to milk-based foods for the development of probiotic-type functional foods. In fact, lactose intolerance, cholesterol content, and allergenic milk proteins are the major drawbacks related to the intake of dairy products, which require the development of new non-dairy probiotic foods. In this contest table olives can be considered an excellent food carrier suitable for delivering probiotics. According to that, we investigate the ability of the potential probiotic *Lactobacillus rhamnosus* H25 and the probiotic LGG strains to survive during the olive fermentation. Moreover, the effect of starters and probiotic strains on the microbiota of green table olives, the volatile compound formation and the sensory profile were also studied (**chapter 3**).

It is worldwide established that probiotic own healthy promoting properties when assumed as food or pharmaceutical supplements. Several clinical study have demonstrated the usefulness of probiotic supplementation for treat gastrointestinal and vaginal diseases. Concerning vaginal microbiota imbalance there are important issue to which great attention must be paid regarding the effect of probiotics on bacterial vaginosis treatment and prevention. In fact, even antibiotic administration is effective in the majority of the cases, a high number of patients have relapses when the assumption is stopped. Therefore, an alternative treatment is desirable. According to that even if the usefulness of probiotic administration, both on BV treatment and prevention, is largely demonstrated no study are

available concerning the effectiveness of *L. rhamnosus* GG (LGG) administration. In the light of the evidence above, the usefulness of LGG, alone or in combination with lactoferrin, to modify the composition and dynamics of vaginal microbiota in bacterial vaginosis patients and to restore the vaginal microbiota homeostasis condition were investigated (**chapter 4**). *Lactobacillus rhamnosus* GG is considered a helpful probiotic strains for gastrointestinal disbiosis treatment. So, we presumed its ability to reduce gastrointestinal and cutaneous symptoms in Systemic Nickel Allergy Syndrome (SNAS) patients and to increase patient's quality of life. Up to now, no information is available concerning the intestinal microbiota composition in SNAS patients and its relation with gastrointestinal symptoms. According to that, the intestinal microbiota of Systemic Nickel Allergy Syndrome patients was characterized, in order to assess the possible existence of a potential syndrome specific microbiota, and the effect of LGG administration in association with the desensitization treatment were studied (**chapter 5**).

REFERENCES

1. Alegre I., Viñas I., Usall J., Anguera M., Abadias M. (2011). Microbiological and physicochemical quality of fresh-cut apple enriched with the probiotic strain *Lactobacillus rhamnosus* GG. Food Microbiology, 28, 59–66.

2. Ankolekar C., Pinto M., Greene D., Shetty K. (2012). In vitro bioassay based screening of antihyperglycemia and antihypertensive activities of Lactobacillus acidophilus fermented pear juice. Innovative Food Science & Emerging Technologies, 13, 221–230.

3. Bergamini C.V., Hynes E.R., Palma S.B., Sabbag N.G., Zalazar C.A. (2009). Proteolytic activity of three probiotic strains in semi-hard cheese as single and mixed cultures: *Lactobacillus acidophilus*, *Lactobacillus paracasei* and *Bifidobacterium lactis*. International Dairy Journal, 19, 467–475.

4. Betoret E., Betoret N., Arilla A., Bennár M., Barrera C., Codoñe, P. (2012). No invasive methodology to produce a probiotic low humid apple snack with potential effect against *Helicobacter pylori*. Journal of Food Engineering, 110, 289–293.

5. Blanchette L., Roy D., Belanger G., Gauthier S.F. (1996). Production of cottage cheese using dressing fermented by bifidobacteria. Journal of Dairy Science, 79, 8–15.

6. Brown M. (2011). Modes of action of probiotics: recent developments. Journal of animal and veterinary advances, 10, 1895-1900.

7. Cardarelli H.R., Buriti F.C.A., Castro I.A., Saad S.M.I. (2008). Inulin and oligofructose improve sensory quality and increase the probiotic viable count in potentially symbiotic petit-suisse cheese. LWT-Food Science and Technology, 41, 1037–1046.

8. Carminati D., Perrone A., Neviani E. (2001). Inhibition of Clostridium sporogenes growth in Mascarpone cheese by co-inoculation with *Streptococcus thermophilus* under conditions of temperature abuse. Food Microbiology, 18, 571–579.

9. Çetin, B. (2011). Production of probiotic mixed pickles (Turşu) and microbiological properties. African Journal of Biotechnology, 10, 14926–14931.

10. Champagne C.P., Gardner N. J., Roy D. (2005). Challenges in the addition of probiotic cultures to foods. Critical Reviews in Food Science and Nutrition, 45, 61–84.

11. Chen T.R., Wei Q.K., Chi Z.X. (2011). Effect of oligosaccharides and isoflavones aglycones in defatted soy meal fermented by *Lactobacillus paracasei* and *Bifidobacterium longum*. African Journal of Microbiology Research, 5, 2011–2018.

12. Chen A., Mustapha A. (2012). Survival of freeze-dried microcapsules of α-galactosidase producing probiotics in a soy bar matrix. Food Microbiology, 30, 68–73.

13. Coda R., Lanera A., Trani A., Gobbetti M., Dicagno R. (2012). Yogurt-like beverages made of a mixture of cereals, soy and grape must: Microbiology, texture, nutritional and sensory properties. International Journal of Food Microbiology, 155, 120–127.

14. Coeuret V., Gueguen M., Vernoux J.P. (2004). In vitro screening of potential probiotic activities of selected lactobacilli isolated from unpasteurized milk products for incorporation into soft cheese. Journal of Dairy Research, 71, 451–460.

15. Commane D., Hughes R., Shortt C., Rowland I. (2005). The potential mechanisms involved in the anti-carcinogenic action of probiotics. Mutation Research, 591, 276−89.

16. Corbo M.R., Albenzio M., De Angelis M., Sevi A., Gobbetti M. (2001). Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. Journal of Dairy Science, 84, 551–561.

17. Damin M.R., Minowa E., Alcˆ Antara M.R., Oliveira M.N. (2008). Effect of cold storage on culture viability and some rheological properties of fermented milk prepared with yogurt and probiotic bacteria. Journal of Texture Studies, 39, 40–55.

18. De Souza C.H.B., Buriti F.C.A., Behrens J.H., Saad S.M.I. (2008). Sensory evaluation of probiotic Minas fresh cheese with *Lactobacillus acidophilus* added solely or in co-culture with a thermophilic starter culture. International Journal of Food Science and Technology, 43, 871–877.

19. De Bellis P., Valerio F., Sisto A., Lonigro S.L., Lavermicocca P. (2010). Probiotic table olives: microbial populations adhering on olives surface in fermentation sets

inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. International Journal of Food Microbiology, 140, 6–13.

20. Doron S., Snydman D. R., Gorbach S. L. (2005). *Lactobacillus* GG: Bacteriology and clinical applications. Gastroenterology Clinics of North America, 34, 483–498.

21. Douillard F.P., Ribbera A., Kant R., Pietilä T.E., Järvinen H.M., Messing M., Randazzo C.L., Paulin L., Laine P., Ritari J., Caggia C., Lähteinen T., Brouns S.J.J., Sotokari R., von Ossowski I., Reunanen J., Palva A, de vos W.M. (2013). Comparive genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains and their comparison with strain GG. PLOS Genetics, 8, 1-15.

22. Ellendersen L.S.N., Granato D., Guergoletto K.B., Wosiacki, G. (2012). Development and sensory profile of a probiotic beverage from apple fermented with *Lactobacillus casei*. Engineering in Life Sciences, 12, 475-485.

23. El-Zayat A., Osman M.M. (2001). The use of probiotics in Tallaga cheese, Egypt. Journal of Dairy Science, 29, 99–106.

24. Espírito-Santo A.P., Perego P., Converti A., Oliveira, M.N. (2011). Influence of food matrices on probiotic viability — A review focusing on the fruity bases. Trends in Food Science and Technology, 22, 377–385.

25. Falagas M.E., Betsi G.I., Stavros A. (2006). Probiotics for prevention of recurrent vulvovaginal candidiasis: a review. Journal of Antimicrobial Chemotherapy, 58, 266–272.

26. Felis G.E. Dellaglio F. (2007). Taxonomy of Lactobacilli and Bifidobacteria. Current Issues Intestinal Microbiology, 8, 44–61.

27. Fernandez M.F., Delgado T., Boris S., Rodriguez A., Barbes C. (2005). A washed-curd goat's cheese as a vehicle for delivery of a potential probiotic bacterium: *Lactobacillus delbrueckii* subsp. *lactis* UO 004. Journal of Food Protection, 68, 2665–2671.

28. Fonteles T.V., Costa M.G.M., Jesus A.L.T., Rodrigues S. (2011). Optimization of the fermentation of cataloupe juice by *Lactobacillus casei* NRRL B-442. Food and Bioprocess Technology, 5, 2819-2826.

29. Food and Agriculture Organization/World Health Organization (FAO/WHO), Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina (2001) (*http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf*).

30. Foroni E., Serafini F., Amidani D., Turroni F., He F., Bottacini F., O'Connell Motherway M., Viappiani A., Zhang Z., Rivetti C., van Sinderen D., Ventura M. (2011). Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. Microbial Cell Factories*,* 10, 1-16.

31. Fritzen-Freire C.B., Muller C.M.O., Laurindo J.O.B., Amboni R.D.M.C., Prudencio E.S. (2010). The effect of direct acidification on the microbiological, physicochemical and sensory properties of probiotic Minas Frescal cheese. International Journal of Dairy Technology, 63, 561–568.

32. Furtado Martins E.M., Ramos A.M., Vanzela E.S.L., Stringheta P.C., de Oliveira Pinto C.L., Martins J.M. (2013). Products of vegetable origin: A new alternative for the consumption of probiotic bacteria. Food Research International, 51, 764–770.

33. Gardiner G., Ross R.P., Collins J.K., Fitzgerald G., Stanton C. (1998). Development of a probiotic Cheddar cheese containing human-derived *Lactobacillus paracasei* strains. Applied Environmental Microbiology, 64, 2192–2199.

34. Gil A. (2013). Third Jesús Culebras Lecture - Molecular biology and clinical nutrition; where do we stand and where do we go? Nutrition Hospitalaria, 28, 241–249.

35. Gobbetti M., Corsetti A., Smacchi E., Zocchetti A., de Angelis M. (1997). Production of Crescenza cheese by incorporation of bifidobacteria. Journal of Dairy Science, 81, 37–47.

36. Gomes A.M.P., Malcata F.X., Klaver F.A.M., Grande H.J. (1995). Incorporation of *Bifidobacterium* spp. strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. Netherlands Milk Dairy Journal, 49, 71–95.

37. Gomes A.M.P., Malcata F.X. (1998). Development of probiotic cheese manufactured from goat milk: response surface analysis via technological manipulation. Journal of Dairy Science, 81, 1492–1507.

38. Gorbach S.L. (1996). The discovery of *Lactobacillus* GG. Nutrition Today, 31, 1S-5S.

39. Gourbeyre P., Denery S., Bodinier M. (2011). Probiotics, prebiotics, and symbiotics: impact on the gut immune system and allergic reactions. Journal of Leukocyte Biology, 89, 685-695.

40. Hamilton-Miller J.M. (2004). Probiotics and prebiotics in the elderly. Postgraduate Medical Journal, 80, 447-451.

41. Hathout A.S., Mohamed S.R., El-Nekeety A.A, Hassan N.S., Aly S.E., Abdel-Wahhab M.A. (2011). Ability of *Lactobacillus casei* and *Lactobacillus reuteri* to protect against oxidative stress in rats fed aflatoxins-contaminated diet. Toxicon, 58, 179–186.

42. Hawrelak J.A., Whitten D.L., Myers S.P. (2005). Is *Lactobacillus rhamnosus* GG effective in preventing the onset of antibiotic-associated diarrhoea: a systematic review. Digestion, 72, 51-56.

43. Hoorde K.V., Leuven I.V., Dirinck P., Heyndrickx M., Coudijzer K., Vandamme P., Huys G. (2010). Selection, application and monitoring of *Lactobacillus paracasei* strains as adjunct cultures in the production of Gouda-type cheeses. International Journal of Food Microbiology, 144, 226–235.

44. Huang J.S., Bousvaros A., Lee J.W., Diaz A., Davidson E.J. (2002). Efficacy of probiotic use in acute diarrhoea in children: a meta-analysis. Digestive Diseases Sciences, 47, 2625-2634.

45. Hugo A.A., De Antoni G.L., Perez P.F. (2006). *Lactobacillus delbrueckii* subsp *lactis* strain CIDCA 133 inhibits nitrate reductase activity of *Escherichia coli*. International Journal of Food Microbiology, 111, 191–196.

46. Hurtado Reguant C., Bordons A., Rozès N. (2012). Lactic acid bacteria from fermented table olives. Food Microbiology, 31, 1–8.

47. Isolauri E., Kirjavainen P.V., Salminen S. (2002). Probiotics: a role in the treatment of intestinal infection and inflammation? Gut, 50, 54–59.

48. Jood S., Khetarpaul N., Goyal R. (2012). Efficacy of barley based probiotic food mixture in treatment of pathogenic *E.coli* induced diarrhoea in mice. Journal of Food Science and Technology, 49, 200–206.

49. Kankainen M., Paulin L., Tynkkynen S., von Ossowski I., Reunanen J., Partanen P., Satokari R., Vesterlund S., Hendrickx A.P., Lebeer S., De Keersmaecker S.C.J., Vanderleyden J., Hämäläinen T., Laukkanen S., Salovuori N., Ritari J., Alatalo E., Korpela R., Mattila-Sandholm T., Lassig A., Hatakka K., Kinnunen K.T., Karjalainen H., Saxelin M., Laakso K., Surakka A., Palva A., Salusjärvi T., Auvinen P., de Vos W.M. (2009). Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. Proceedings of the National Academy of Science of the U.S.A. 106, 17193–17198.

50. Klatte T., Evans L., Whitehead R.N., Cole J.A. (2011) Four PCR primers necessary for the detection of periplasmic nitrate reductase genes in all groups of Proteobacteria and in environmental DNA. Biochemical Society Transactions, 39, 321–326.

51. Komatsu T.R., Buriti F.C.A., Saad S.M.I. (2008). Overcoming hurdles through innovation, persistence and creativeness in the development of probiotic foods. Brazilian Journal of Pharmaceutical Sciences, 44, 329–347.

52. Kourkoutas Y., Kanellaki M., & Koutinas A.A. (2006). Apple pieces as immobilization support of various microorganisms. LWT- Food Science and Technology, 39, 980–986.

53. Lebeer S., Claes I., Tytgat H.L.P., Verhoeven T.L., Marien E., von Ossowski I., Reunanen J., Palva A., de Vos W.M., de Keersmaecker S.C.J., Vanderleyden J. (2012). Functional analysis of *Lactobacillus rhamnosus* GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. Applied Environmental Microbiology, 78, 185–193.

54. LeBlanc J.G., Laiño J.E., Juarez del Valle M., Vannini V., van Sinderen D., Taranto M.P., Font de Valdez G., Savoy de Giori G., Sesma F. (2011). B-Group vitamin production by

lactic acid bacteria – current knowledge and potential applications. Journal of Applied Microbiology, 111, 1297–1309.

55. Lee S. Y., Ganesan P., Ahn J., Kwak H. S. (2011). *Lactobacillus acidophilus* fermented yam (Dioscorea opposita Thunb.) and its preventive effects on gastric lesion. Food Science and Biotechnology, 20, 927–932.

56. Levri KM, Ketvertis K, Deramo M, Merenstein JH, D'Amico F. (2005). Do probiotics reduce adult lactose intolerance? A systematic review. Journal of Family Practice, 54, 613-620.

57. Lourens-Hattingh A.,Viljoen B.C. (2001). Yogurt as probiotic carrier food. International Dairy Journal, 11, 1–17.

58. Lucas A., Sodini I., Monnet C., Jolivet P., Corrieu G. (2004). Probiotic cell counts and acidification in fermented milks supplemented with milk protein hydrolysates. International Dairy Journal, 14, 47–53.

59. Martarelli D., Verdenelli M.C., Scuri S., Cocchioni M., Silvi S., Cecchini C., Pompei P. (2011). Effect of a probiotic intake on oxidant and antioxidant parameters in plasma of athletes during intense exercise training. Current Microbiology, 62, 1689-1696.

60. Masuda T., Yamanari R., Itoh T. (2005). The trial for production of fresh cheese incorporated probiotic *Lactobacillus acidophilus* group lactic acid bacteria. Milchwissenschaft, 60, 167–71.

61. McBrearty S., Ross R.P., Fitzgerald G.F., Collins J.K., Wallace J.M., Stanton C. (2001). Influence of two commercially available bifidobacteria cultures on Cheddar cheese quality. International Dairy Journal, 11, 599–610.

62. Meyrand M., Guillot A., Goin M., Furlan S., Armalyte J., Kulakauskas S., Cortes-Perez N.G., Thomas G., Chat S., Péchoux C., Dupres V., Hols P., Dufrêne Y.F., Trugnan G., Chapot-Chartier M.P. (2013). Surface proteome analysis of a natural isolate of *Lactococcus lactis* reveals the presence of pili able to bind human intestinal epithelial cells. Molecular and Cellular Proteomics, 12, 3935–3947.

63. Milesi M.M., Vinderola C.G., Sabbag N., Meinardi C.A., Hynes E. (2009). Influence on cheese proteolysis and sensory characteristics of non-starter lactobacilli strains with probiotic potential. Food Research International, 42, 1186–1196.

64. Mousavi Z.E., Mousavi S.M., Razavi S.H., Emam-Djomeh Z., Kiani H. (2011). Fermentation of pomegranate juice by probiotic lactic acid bactéria. World Journal of Microbiology and Biotechnology, 27, 123–128.

65. Murad H.A., Sadek Z.I., Fathy F.A. (1998). Production of bifidus Kariesh cheese. Dtsch Lebensm–Rundsch, 94, 409–412.

66. Nicolesco C.L., Buruleanu L.C. (2010). Correlation of some substrate parameters in growing *Lactobacillus acidophilus* on vegetable and fruit cocktail juices. Bulletin UASVM Agriculture, 67, 352–359.

67. Nualkaekul S., Charalampopoulos D. (2011). Survival of *Lactobacillus plantarum* in model solutions and fruit juices. International Journal of Food Microbiology, 146, 111–117.

68. O'Connell Motherway M., Zomer A., Leahy S.C., Reunanen J., Bottacini F., Claesson M.J., O'Brien F., Flynn K., Casey P.G., Muno J.A.M., Kearney B., Houston A.M., O'Mahony C., Higgins D.G., Shanahan F., Palva A., de Vos W.M., Fit gerald G.F., Ventura M., O'Toole P.W., van Sinderen D. (2011). Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proceedings of the National Academy of Science of the U.S.A., 108, 11217–11222.

69. Oliveira M. N., Sodini I., Remeuf F., Corrieu G. (2001). Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. International Dairy Journal, 11, 935–942.

70. Ong L., Henriksson A., Shah N.P. (2006). Development of probiotic Cheddar cheese containing *Lb. acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal, 16, 446–456.

71. Ong L., Henriksson A., Shah N.P. (2007). Chemical analysis and sensory evaluation of Cheddar cheese produced with *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* or *Bifidobacterium* sp. International Dairy Journal, 17, 937–945.

72. Patel A.K., Singhania R.R., Pandey A., Chincholkar S.B. (2010) Probiotic Bile Salt Hydrolase: Current Developments and Perspectives. Applied Biochemistry and Biotechnology, 162, 166-180.

73. Pereira L.C., de Souza C.H.B., Behrens J.H., Saad S.M.I. (2010). *Lactobacillus acidophilus* and *Bifidobacterium* sp. in co-culture improve sensory acceptance of potentially probiotic petit-suisse cheese. Acta Alimentaria, 39, 265–276.

74. Pereira A.L.F., Maciel T.C., Rodrigues S. (2011). Probiotic beverage from cashew apple juice fermented with *Lactobacillus casei*. Food Research International, 44, 1276–1283

75. Peres C. M., Peres C., Hernández-Mendoza A., Malcata F. X. (2012). Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria— With an emphasis on table olives. Trends in Food Science and Technology, 26, 31–42.

76. Phillips M., Kailasapathy K., Tran L. (2006). Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei*, *L. paracasei* and *L. rhamnosus*) in Cheddar cheese. International Journal of Food Microbiology, 108, 276–280.

77. Plessas S., Bosnea L., Alexopoulos A., Bezirtzoglou E. (2012). Potential effects of probiotics in cheese and yogurt production: A review. Engineering Life Sciences, 12, 433–440.

78. Pompei A., Cordisco L., Amaretti A., Zanoni S., Matteuzzi D., Rossi M. (2007). Folate Production by Bifidobacteria as a Potential Probiotic Property. Applied Environmental Microbiology, 73, 179-185.

79. Possemiers S., Marzorati M., Verstraete W., Wiele T.V. (2010). Bacteria and chocolate: A successful combination for probiotic delivery. International Journal of Food Microbiology, 141, 97–103.

80. Prado F.C., Parada J.L., Pandey A., Soccol C.R. (2008). Trends in non-dairy probiotic beverages. Food Research International, 41, 111–123.

81. Ranadheera R.D.C.S., Baines S.K., Adams M.C. (2010). Importance of food in probiotic efficacy. Food Research International, 43, 1–7.

82. Rathore S., Salmerón I., Pandiella S.S. (2012). Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. Food Microbiology, 30, 239–244.

83. Rautava S., Kalliomäki M., Isolauri E. (2002). Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. Journal of Allergy and Clinical Immunology, 109, 119–121.

84. Rautava S., Kalliomäki M., Isolauri E. (2005). New therapeutic strategy for combating the increasing burden of allergic disease: Probiotics-A Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report. Journal of Allergy and Clinical Immunology, 116, 31-37.

85. Reid G., Charbonneau D., Erb J., Kochanowski B., Beuerman D., Poehner R., Bruce A.W. (2003). Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunology and Medical Microbiology, 35, 131-134.

86. Reid G., Jass J., Sebulsky M.T., McCormick J.K. (2003). Potential uses of probiotics in clinical practice. Clinical Microbiology Reviews, 16, 658-672.

87. Reid G., Sanders M.E., Gaskins H.R., Gibson G.R., Mercenier A., Rastall R. (2003). New scientific paradigms for probiotics and prebiotics. Journal of Clinical Gastroenterology, 37, 105–118.

88. Reunanen J., von Ossowski I., Hendrickx A.P., Palva A., de Vos W.M. (2012). Characterization of the SpaCBA pilus fibers in the probiotic *Lactobacillus rhamnosus* GG. Applied Environmental Microbiology, 78, 2337–2344.

89. Rodgers S. (2007). Incorporation of probiotics in food service products: An exploratory study. Journal of Foodservice, 18, 108–118.

90. Rößle C., Auty M.A.E., Brunton N., Gormley R.T., Butler F. (2010). Evaluation of fresh-cut apple slices enriched with probiotic bacteria. Innovative Food Science and Emerging Technologies, 11, 203–209.

91. Rößle C., Brunton N., Gormley R.T., Ross P.R., Butler F. (2010). Development of potentially symbiotic fresh-cut apple slices. Journal of Functional Foods, 2, 245–254.

92. Roy D., Mainville I., Mondou F. (1997). Selective enumeration and survival of bifidobacteria in fresh cheese. International Dairy Journal, 7, 785–793.

93. Ryhanen E.L., Pihlanto-Leppala A., Pahkala E. (2001). A new type of ripened, low-fat cheese with bioactive properties. International Dairy Journal, 11, 441–447.

94. Saad S.M.I. (2006). Probiotics and prebiotics: The state of the art. Brazilian Journal of Pharmaceutical Sciences, 42, 1–16.

95. Saarela M.H. (2007). Probiotic technology maintaining viability and stability. Agro Food Industry Hi Tech, 18, 19–21.

96. Savino F., Cresi F., Pautasso S., Palumeri E., Tullio V., Roana J., Silvestro L., Oggero R. (2004). Intestinal microflora in breastfed colicky and non-colicky infants. Acta Paediatrica, 93, 825–829.

97. Savino F., Nanni G.E., Maccario S., Costamagna M., Oggero R., Silvestro L. (2004). Breast-fed Infants have Higher Leptin Values than Formula-fed Infants in the First Four Months of Life. Journal of Paediatric Endocrinology and Metabolism, 17, 1527–1532.

98. Savino F., Tarasco V. (2010). New treatments for infant colic. Current Opinion in Pediatrics, 22, 791–797.

99. Saw L.K., Chen S., Wong S.H., Tan S.A., Goh K.K.T. (2011). Fermentation of tropical fruit juices by lactic acid bacteria. 12th ASEAN Food Conference, 16–18 June, Kangkok, Thailand, 80–87.

100. Shah N.P., Lankaputhra W.E.V., Britz M.L., Kyle W.S.A. (1995). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. International Dairy Journal, 5, 515–521.

101. Shanahan F. (2004). Probiotics in inflammatory bowel disease--therapeutic rationale and role. Advanced Drug Delivery Reviews, 56, 809-818.

102. Sharp M.D., McMahon D.J., Roadbent J.R. (2008). Comparative evaluation of yogurt and low-fat Cheddar cheese as delivery media for probiotic *Lactobacillus casei*. Journal of Food Science, 73, 375–377.

103. Sheehan V. M., Ross P., Fitzgerald G.F. (2007). Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. Innovative Food Science and Emerging Technologies, 8, 279–284.

104. Sheela T., Suganya R.S. (2012). Studies on anti-diarrhoeal activity of symbiotic plums juice. International Journal of Scientific and Research Publications, 2, 1–5.

105. Soccol C.R., Vandenberghe L.P.S., Spier M.R., Medeiros A.B.P., Yamaguishi C.T., Lindner J.D. (2010). The potential of probiotics. Food Technology Biotechnology, 48, 413–434.

106. Souza C.H.B., Buriti F.C.A., Behrens J.H., Saad S.M.I. (2008). Sensory evaluation of probiotic Minas fresh cheese with *Lactobacillus acidophilus* added solely or in co-culture with a thermophilic starter culture. International Journal of Food Science and Technology, 43, 871–877.

107. Starling S [Internet]. US functional foods market to grow 21 per cent by 2015; 2010 [Accessed 2010 Dec 08]. Available from: http://www.nutraingredients-usa.com/ Consumer-Trends/ US-functional-foods-market-to-grow-21-per-cent-by-2015.

108. Strozzi G., Paolo B.S., Mogna L.B.S. (2008).Quantification of folic acid in human feces after administration of Bifidobacterium probiotic strains. Journal of Clinical Gastroenterology, 42, 179-182.

109. Sung V., Hiscock H., Tang M.L.K., MensahF.K., Nation M.L., Satzke C., Heine R.G., Stock A., Barr R.G., Wake M. (2014). Treating infant colic with the probiotic *Lactobacillus reuteri*: double blind, placebo controlled randomised trial. British Medical Journal, 348, 1-11.

110. Szajewska H., Ruszczyński M., Radzikowski A. (2006). Probiotics in the prevention of antibiotic-associated diarrhoea in children: a meta-analysis of randomized controlled trials. Journal of Pediatrics, 149, 367-372.

111. Tanguler H., Erten H. (2012). Occurrence and growth of lactic acid bacteria species during the fermentation of shalgam (salgam), a traditional Turkish fermented beverage. Food Science and Technology, 46, 36–41.

112. Tapia M.S., Rojas-Graü M.A., Rodríguez F.J., Ramírez J., Carmona A., Martin-Belloso, O. (2007). Alginate and gellan based edible films for probiotic coatings on fresh-cut fruits. Journal of Food Science, 72, 190–196.

113. Turroni F., Serafini F., Foroni E., Duranti S., O'Connell Motherway M., Taverniti V., Mangifesta M., Milani C., Viappiani A., Roversi T., Sánchez B., Santoni A., Gioiosa L., Ferrarini A., Delledonne M., Margolles A., Piazza L., Palanza P., Bolchi A., Guglielmetti S., van Sinderen D., Ventura M. (2013). Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host interactions. Proceedings of the National Academy of Science of the U.S.A., 110, 11151–11156.

114. Vélez M.P., Petrova M.I., Lebeer S., Verhoeven T.L.A., Claes I., Lambrichts I., Tynkkynen S., Vanderleyden,J., De Keersmaecker S.C.J. (2010). Characterization of MabA, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation. *FEMS* Immunology and Medical Microbiology, 59, 386–398.

115. Vergara C.M.A.C., Honorato T.L., Maia G.A., Rodrigues, S. (2010). Prebiotic effect of fermented cashew apple (Anacardium occidentale L) juice. Food Science and Technology, 43, 141–145.

116. Vinderola C.G., Prosello W., Ghiberto D., Reinheimer J.A. (2000). Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and non-probiotic microflora in Argentinian Fresco cheese. Journal of Dairy Science, 83, 1905–1911.

117. Vinderola C.G., Prosello W., Molinari F., Ghiberto D., Reinheimer J.A. (2009). Growth of *Lactobacillus paracasei* A13 in Argentinian probiotic cheese and its impact on the characteristics of the product. International Journal of Food Microbiology, 135, 171–174.

118. Von Ossowski I., Reunanen J., Satokari R., Vesterlund S., Kankainen M., Huhtinen H., Tynkkynen S., Salminen S., de Vos W.M., Palva A. (2010). Mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pili subunits. Applied Environmental Microbiology, 76, 2049–2057.

119. Walker A.W. (2008). Mechanisms of action of probiotics. Clinical infectious diseases, 46, 87-91.

120. Yoon K. Y., Woodams E. E., Hang Y. D. (2004). Probiotication of tomato juice by lactic acid bacteria. Journal of Microbiology, 42, 315–318.

121. Yoon K. Y., Woodams E. E., Hang Y. D. (2005). Fermentation of beet juice by beneficial lactic acid bacteria. Food Science and Technology, 38, 73–75.

122. Yoon K. Y., Woodams E. E., Hang Y. D. (2006). Production of probiotic cabbage juice by lactic acid bacteria. Bioresource Technology, 97, 1427–1430.

123. Yu Z.H., Zhang X., Li S.Y., Li C.Y., Li D., Yang, Z.N. (2012). In vitro evaluation of probiotic properties of *Lactobacillus plantarum* strains isolated from Chinese Sauerkraut. African Journal of Biotechnology, 11, 4868–4875.

Chapter 2

Lactobacillus rhamnosus in Pecorino Siciliano production and ripening

Randazzo C.L., Pino A., Russo N., Van Hoorde K., Carpino S., Caggia C.

Manuscript for publication in preparation

***Lactobacillus rhamnosus* H25 and *Lactobacillus paracasei* N24 for probiotic Pecorino Siciliano cheese production: study of probiotics survival during production, ripening and through the human gastrointestinal tract.**

INTRODUCTION

The profound changes in the society lifestyles of the last decades have made consumers more conscious regard connection among health, longevity and diet, orienting their choices towards new food models, called functional foods (33, 34, 13, 49). The major development in functional foods is related to food containing probiotic cultures, mainly lactic acid bacteria or bifidobacteria. According to FAO/WHO which defined probiotics as "*Live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2001), a probiotic food is a processed product which contains viable probiotic microorganisms in a suitable matrix and in sufficient cell density (39). The matrix has a large impact on the probiotic viability. For instance, a minimum of 10^7 CFU/mL or g and even higher numbers per daily portion of probiotic bacteria must be present in a product to obtain a proper delivery in the gastrointestinal (GI) tract and to retain their health benefits (18, 2, 22). It is already well established that cheese represents an excellent food probiotic carrier, able to support bacterial viability and stability during storage (8). Cheese creates a buffer against the high acidic conditions in the GI tract, establishing a more favorable environment for probiotic delivery throughout the gastric transit. Moreover, the dense matrix and relatively high fat content of cheese may offer additional protection to probiotic bacteria in the stomach (18, 1, 35, 41). Nevertheless, during manufacturing and cheese ripening several hurdles, such as the amount of inoculum, redox potential, pH, salting, ripening, packaging, and microbiota competition directly influence the maintenance of the functional activities of probiotic

bacteria. In particular, the fall of water activity throughout ripening and microbiota competition create a hostile and stressful environment for probiotic cultures (20). Hence, due to its manufacturing process, fresh cheese appears to be ideally suited to serve as a carrier for probiotic bacteria (21, 18). Numerous scientific papers, which report the development of fresh cheeses containing potentially probiotic strains have been already published (11, 43, 15, 24, 17, 50, 51, 6). Differently, only few data are available on the viability of probiotic strains during cheese ripening. The aim of the present work was to study the viability of two promising probiotic lactobacilli (*Lactobacillus rhamnosus* H25 and *Lactobacillus casei* N24) strains, used in mixed culture, during the ripening of Pecorino Siciliano cheese, made from pasteurized ewe's milk with the addition of commercial starter cultures, through a combination of culture-dependent and independent approaches. Moreover, a pilot human trial was carried out in order to evaluate the H25 and N24 ability to survive passage through the GI tract and to maintain their persistence.

MATERIAL AND METHODS

Bacterial strains and growth conditions

The potential probiotic strains *Lactobacillus rhamnosus* (H25) and *Lactobacillus paracasei* (N24), previously isolated from Pecorino Crotonese cheese (31), identified for phenotypical and genotypical features (29) and analyzed for their probiotic properties (32) were selected and used for Pecorino Siciliano cheese making. The strains kept at -80°C in glycerol, were sub-cultured twice consecutively at 37°C overnight in 12% reconstituted skim milk supplemented with 0.25% (w/v) of yeast powder and 0.20% (w/v) of glucose.

Heat resistance

Lactobacillus rhamnosus (H25) and *Lactobacillus paracasei* (N24) were studied, separately, for heat resistance following the protocol of Minervini et al., 2012 (25). In detail, each strain was cultivated in MRS broth at the optimum temperature until it reached the stationary phase of growth (18 h; optical density at 620 nm=2.44). Cells were harvested by centrifugation at 9,000 × *g* for 10 min at 4°C, washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0), and re-suspended in sterile distilled water. The suspension was centrifuged (9,000 × *g* for 10 min at 4°C) and the cells re-suspended in reconstituted skim milk (Oxoid, Milan, Italy) to a final cell density of 9.8 $\log_{10}$ CFU/ml. Aliquots (0.5 ml) were transferred to capillary glass tubes and heated in a water bath at 55°C for 10 minutes. After heat treatment, the samples were put on ice for 5 min, diluted, plated on MRS agar and incubated at 37°C for 48-96 h. For each strain, the heat resistance test was carried out in duplicate and the average and standard deviation were calculated.

Heat adaptation

Heat adaptation was performed for each strain following the protocol of Minervini et al., 2012 (25). The strains were harvested at stationary phase (18 h), washed and re-suspended in reconstituted skim milk at the final density of 9.8 $\log_{10}$ CFU/ml. To induce heat adaptation, cell suspensions, for each strain, were incubated for 20, 30, 40, and 60 min at 42°C. Control cells were incubated for 20, 30, 40, and 60 min at 37°C. After incubation, control and adapted cells were treated at 55°C for 10 min then the cultivable cells were enumerated on MRS agar. Two separate experiment were performed and the average value was calculated. The decimal reduction time (D-value, the time taken to reduce the cell numbers by 1 $\log_{10}$ cycle) was

evaluated after heat treatment. In detail, the adapted cells (42°C for 30 min) were treated at 55°C for 5 to 120 min and their survival rate after planting was calculated as average of three separate experiments performed for each strain.

Coagulant and acidifying ability

The strains H25 and N24 were tested for coagulant and acidifying ability singularly and in mixture.

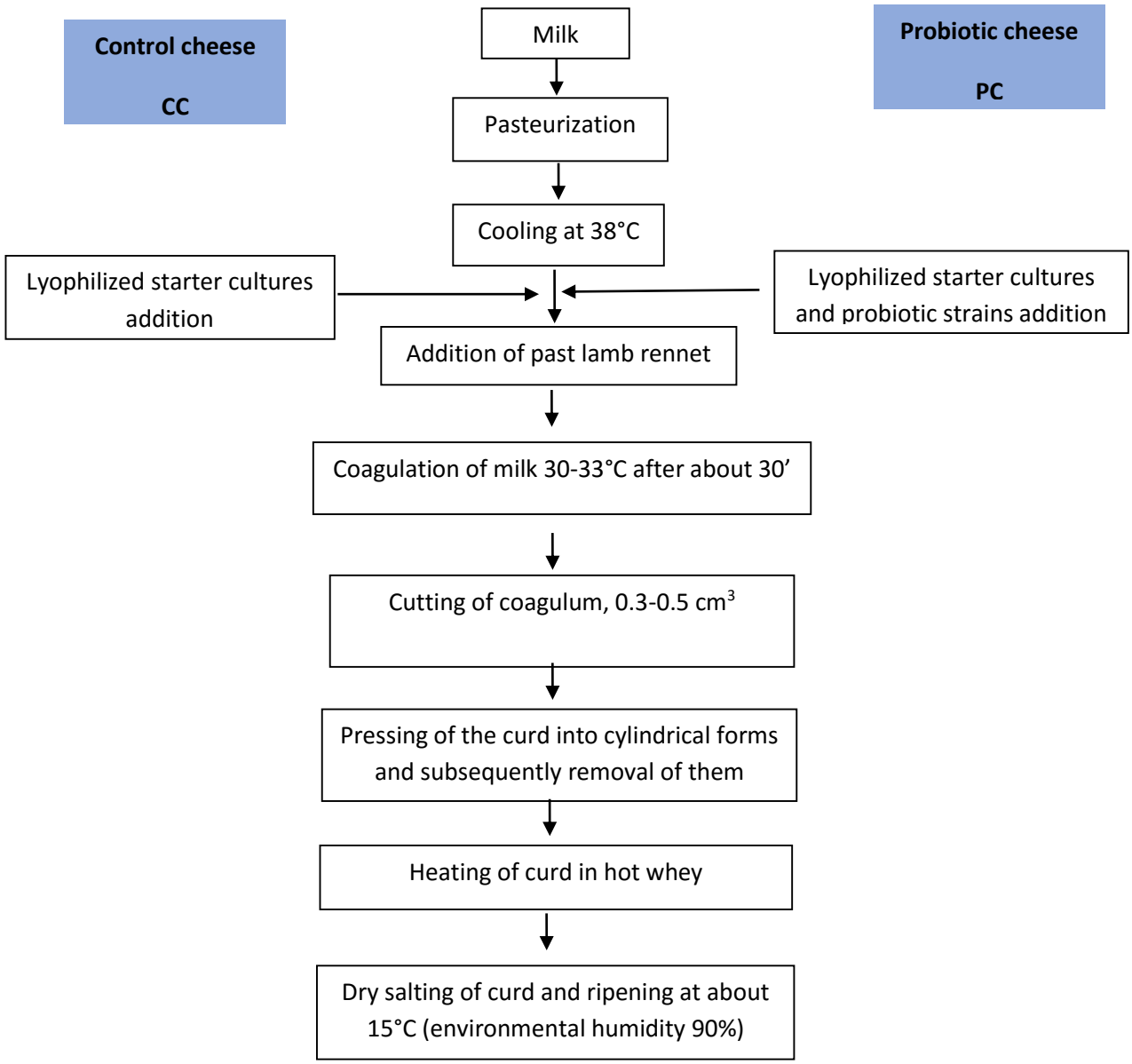
Three different mixtures of the potential probiotic strains (M1, ratio1:1; M2, ratio 1:2 and M3 ratio 2:1) were performed. In detail, adequate amounts of each overnight culture were inoculated into both skim and UHT milk to reach the cell density of 10^8-10^9 CFU/ml then incubated for 18 h at room temperature (22°C). After incubation, coagulant ability was evaluated by visualizing the clot formation, and acidifying ability was estimated by calculation the pH using a pHmeter (Instrument XSPH 510). Moreover, the mixture that exhibited the better performance was tested in combination with commercial starter cultures usually used for the Pecorino Siciliano cheese making. Three different combinations between probiotic strains and starter cultures (C1 ratio 1:1, C2 ratio 1:2, and C3 ratio 2:1) were performed. In detail, the starter strains, from lyophilized stock cultures, were activated by growing at least twice consecutively for 24 h at 32°C, reaching the 10^8 CFU/ml cell density, and inoculated with the probiotics mixture both in skim and in UHT milk then incubated and tested as previously reported. The starter culture (Sacco srl, Milan, Italy) contained the followed strains: *Lactococcus lactis* ssp. *lactis* biovar *diacetilactis, Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris* and *Streptococcus thermophilus*.

The analysis were carried out in duplicate.

Experimental probiotic strains culture and cheese making

Aliquots of the overnight cultures of both H25 and N24 strains, enough to attain cell density of 10^9 CFU/ml in the milk used for probiotic cheese manufacture, were centrifuged for 20 min at 8000 g under refrigeration (4°C). The pellets were washed twice with 0.1 M sodium phosphate buffer (pH 7), re-suspended on 50 ml of UHT milk and then incubated at 32°C for 16-18 h. Adequate amounts of each pre-culture were transferred, in a ratio 1:1, into 5 l of UHT milk and incubated under the same condition previously reported. Pecorino Siciliano cheese making was carried out at La Cava srl dairy factory (Randazzo, Catania, Italy), according to the procedure illustrated in Figure 2.1. Overall, two separated cheese making were performed and two different types of Pecorino Siciliano were manufactured: probiotic cheese (PC) and control cheese (CC). 250 l of milk, for each production, were pasteurized at 72°C for 15 s, cooled to 38°C and inoculated with lyophilized starter cultures alone (CC) or in association with experimental probiotic strains culture (PC).

Figure 2.1 Flow chart of Pecorino Siciliano cheese making.



Microbiological analysis

Microbial counts

Probiotic and control cheeses (20 g) were analyzed at 0, 30, 60, 90, 120 and 180 days of ripening. Two diametrically opposed samples including the cheese core and surface were putted in a stomacher bag, blended for 3-5 min with a Stomacher Lab Blender 400 (International PBI S.p.A Milan, Italy) then serially diluted using sterile quarter-strength Ringer's solution (QRS), and plated in duplicate. Microbiological counts were performed on the following agar media and conditions: Plate Count Agar (PCA) (Sigma,Milan, Italy), incubated at 30°C for 72 h, to determine the viable mesophilic counts; MRS, anaerobically incubated at 37 °C for 48 h, for LAB counts; SL-Rogosa agar incubated at 37°C for 3 days anaerobically; Sabouraud Dextrose Agar (SDA, Oxoid) supplemented with chloramphenicol (0.05 g l−1) and incubated at 25 °C for 4 days for yeast counts; LM17 agar (Oxoid), added to 0.17 g/L of cycloheximide (Oxoid), incubated at 32°C and 45°C for mesophilic and thermophilic lactococci respectively; Kanamycin Aesculin Agar (KAA) incubated at 32°C for 48 h for enterococci; and Violet Red Bile Glucose Agar (VRBGA, Difco, Italy), aerobically incubated at 37 °C for 24 h, for total Enterobacteriaceae counts. The analysis were carried out in duplicate and the results were expressed as $\log_{10}$ CFU per g (log CFU g^{-1}).

Isolation and identification of Lactobacilli

From each SL-Rogosa plate of Probiotic cheese (PC) samples at 60, 120, and 180 days of ripening, 20 colonies were randomly selected, purified, checked for catalase activity and Gram reaction, and microscopically examined prior storing in liquid culture using 20% glycerol at−80°C. Total genomic DNA was extracted from overnight cultures following the method

described by Gala et al. (2008) (16). DNA concentration and DNA quality were assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). Lactobacilli isolates were identified at species level by *tuf* gene multiplex PCR (48). PCR reactions were carried out in a final volume of 50 µl containing 25 ng of template DNA, 2.5 U of Taq DNA polymerase (Invitrogen, Italy), 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 µM each dNTPs, 10 pmol of each primer PAR (5-GACGGTTAAGATTGGTGAC-3), CAS (5-ACTGAAGGCGACA AGGA-3), and RHA (5-GCGTCAGGTTGGTGTTG-3), 50 pmol of primer CPR (5-CAANTGGATNGAACCTGGCTTT-3). Amplification reactions were performed by using a GeneAmp PCR System 2400 (Applied Biosystems, Norwalk, CT, USA), with the following temperature profiles: 1 cycle at 5 °C for 5 min; 30 cycles at 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 1.5 min; 1 cycle at 72 °C for 7 min. The PCR products were resolved by electrophoresis using 1% agarose gel in TBE buffer (89mMTris–borate, 89mMboric acid, 2mMEDTA; pH 8.0) and visualized after staining with Gel Red Nucleic Acid Stain (Biotium, Italy).

Total DNA extraction from cheeses and PCR amplification

Cheese samples, taken during cheese ripening (0-180 days), were collected for direct DNA extraction, as previously reported by Randazzo et al., 2002 (30). DNA concentration and DNA quality were assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). The universal primers HDA1-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3'), amplifying the V2 to V3 region of 16S rDNA, were used. PCR amplification was performed with the KIT 5-PRIME MasterMix (Eppendorf, Italy). Reaction mixtures consisted of 75 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM Mg2+, 10 mM each of the

four deoxynucleoside triphosphates (dNTP), 1.255 U/mL of Taq polymerase, 10 pMol of each primer, and 1 µl of appropriately diluted template DNA in a final volume of 50µl. The thermocycle program consisted of the following time and temperature profile: 94°C for 2 min; 30 cycles of 93°C for 30 s, 57°C for 30 s, 72°C for 30 s; and 72°C for 2 min. The 40-nucleotide GC-rich sequence at the 5' end of primers HDA1 improves the detection of sequence variations of amplified DNA fragments by subsequent DGGE (30). PCR products were visualized by UV transillumination and photographed by Mitsubishi Digital P91 video coy processor.

DGGE analysis of PCR amplicons

To investigate the dominant bacterial communities DGGE analysis of PCR amplicons was performed on the Dcode System apparatus (BioRad, Hercules, CA, USA) as previously described (26). Samples were applied to an 8% (w/v) polyacrylamide gel (acrylamide/bisacrylamide 37.5:1) in 0.5X TAE buffer (2 M Tris-base, 1 M glacial acetic acid, 50 M EDTA; pH 8.0). 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) formamide. Electrophoresis was performed at a constant voltage of 85 V and at a temperature of 60 -C for 16 h. The DNA bands were visualized by silver staining and were developed as previously described (38).

Cloning and sequencing of 16S rRNA gene in plasmid inserts

Clone libraries of the 16S rRNA gene amplicons from PC and CC cheeses 60, 120, and 180 days old 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 of the transformants were amplified using the primers pair U968-GC and L1401 and their mobility was compared to the rDNA-derived patterns of 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 cheese DNA patterns were selected for sequence analysis. 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. Sequences with a percentage identity of 98% or greater were considered to belong to the same species.

SmartNose analysis

Probiotic and control cheeses at 30, 60, 120 and 180 days of ripening were subjected to the volatile organic components (VOCs) detection performed with an electronic nose. The SMartNose system (from LDZ, CH-2074 Marin-Epagnier) used incorporates the Combi Pal auto sampler CTC Analytics AG (CTC Combi Pal with the Cycle Composer software), a high-sensitivity quadrupole mass spectrometer (Inficon AG) with a ionic mass detection ranging from 1 to 200 amu, and a user-friendly multivariate analysis software (SMart Nose 1.51) for data acquisition and analysis. Five gr of each cheese samples were put into 20-mL vials (adapted for the Combi Pal autosampler) closed with a butyl/PTFE septum and a cap. The cheese samples were randomly placed in the auto sampler trays to avoid biases due to external factors and the so-called “memory effect”. Three replicates were measured for each sample. The main operating conditions were as follow: incubation temperature at 45° C; incubation time of 30 min;

injection volume of 2.5 mL; syringe temperature at 100° C; injector temperature at 160° C; nitrogen as purge gas, with a purge flow of 200 mL/min; EI ionization mode at 70 eV; mass spectrometer scan speed of 0.5 s/mass; mass range of 10–160 amu; SEM voltage at 1540. The total acquisition time was set to 170 s so that 3 cycles were measured for each injection (7). All data set transformations were carried out using the software supplied with the SMartNose. The mean value of the three cycles was calculated, and the processed data set was normalised using the atomic ion of argon (m/z = 40) from air. This mass-to-charge ratio is subjected to no contamination from other compounds and the concentration of the gas in the headspace can be considered as constant. Such a normalization makes it possible to correct the drift both within a single series of measurements and between different series. Then, a Principal Components Analysis (PCA) was performed.

Sensory analysis

In order to evaluate the sensory differences among PC and CC cheeses, the sensory profile method UNI, 10957- 2003 (45), the triangle test and the test of preference were performed on cheeses samples at 60, 120 and 180 days of ripening. A panel of 12 judges (7 females and 5 males, aged between 24 and 40 years) was trained in preliminary sessions in order to develop a common vocabulary for the description of the sensory attributes of cheeses and to familiarize themselves with scales and procedures. For the sensory profile, each attribute term was explained to avoid any doubt about the relevant meaning. On the basis of the frequency of citation (N>60%), twenty descriptors were selected to be inserted in the card: colour, holes, greasiness, compactness, acid, bitter, salt (taste), spicy, odour and taste of: milk, butter, hazelnut, fruits, cheese, off-odour, off-flavour. Random samples were evaluated by assigning

a score between 1 (absence of the sensation) and 9 (extremely intense). Data were acquired by a direct computerized registration system (FIZZ Biosystemes. ver. 2.00 M, Couternon, France). The sensory data for each attribute were submitted to one-way ANOVA by the software package Statgraphics® Centurion XVI (Statpoint Technologies, INC.) using samples as factors. The significance was tested by means of the F-test. To differentiate the samples, the mean values were submitted to the multiple comparison test using the least significant difference (LSD) procedure.

According to the triangle test method three products were presented, two of which were identical and one was different. Panellists had to pick the odd one. Sensory analyses were replicated four times. No information was given to the panellists about the origin of the samples. Results of sensory evaluation were analyzed according to UNI ISO 4120:2004 (46) ($p=1/3$; $p_a=2/3$; $\alpha = 0.05$; $\beta = 0.10$; $p_d= 40$ %; $N=30$). Test of preference was carried out according to the ISO 5495: 2005 (47) and unilateral binomial test ($\pi=0.50$) was applied on preferences expressed on correct answers of the triangle test. The random and equalized distribution of the samples was applied by using the *Compusense* software in order to obtain the right balance of samples combination.

Statistical analysis

All experiments were performed in duplicate and the experimental data were reported as average values and provided with Standard Deviation. Statistical ANOVA ($P < 0.05$) and Duncan tests were performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA). Statistical ANOVA was carried out to evaluate significant differences on bacterial growth on different media during the whole cheese ripening. Moreover, GLM repeated measures procedures, which

provides analysis of variance when the same measurement is made several times on each subject or case, were applied. Specifically, GLM repeated measures analysis was performed in order to evaluate significant differences between control (CC) and probiotic (PC) cheeses.

Human intervention study

Trial design and participant's selection

Ten healthy volunteers (4 men and 6 female, age from 25 to 60 years old, average 42.5) participated to the study. Information on the healthy status as well as about current diet and lifestyle were obtained at the enrollment. Exclusion criteria were: BMI outside the range 20-29 Km/m2, hypertension, diabetes, hypercholesterolemia, hypertriglyceridemia, lactose allergy and intolerance, diagnosed chronic intestinal diseases, systemic and endocrine pathologies. Informed consent was obtained from all volunteers and they were asked to no take antibiotics, probiotics, prebiotics, anti-inflammatory or prokinetic drugs 1 month prior to the study and for the whole duration of the study.

The trial had a duration of 4 weeks and it consisted of four phases: baseline (T1), 1-week probiotic cheese administration (T2), 2-weeks probiotic cheese administration (T3), and 2-weeks follow-up period (T4). Participants integrated their usual lifestyle and dietary intake with Pecorino Siciliano probiotic cheese 180 days-old. Twenty gram of cheese, vacuum packed to obtain a ready-to-eat-product, were daily ingested during the administration periods. Fecal samples were collected during each phase of the study (T1, T2, T3, and T4) and were subjected to microbiological and molecular analysis as followed reported.

Lactobacilli counts

Fresh fecal samples (about 20 gram) were collected in sterile plastic box previously filled with Amies transport medium (1:10 w/v) (BD Italia; Milan, Italy), stored at 4°C in the volunteer's home and delivered to the laboratory within 24 hours after collection. Microbiological analysis were performed, in duplicate, as soon as the fecal samples were received. Serially dilution were made in reduced peptone water (0.1% w/v peptone with 0.05% w/v cysteine-HCl) and decimal dilution were planted in duplicate on SL-Rogosa agar (Oxoid). Plates were incubated in anaerobic jars for 48 h at 37°C; colonies count were obtained and expressed as $\log_{10}$ of the colony-forming units per gram (CFU g^{-1}) of fresh faeces.

Genetic identification of *L. rhamnosus* H25 and *L. paracasei* N24 strains

From each plate and each sampling time, the 20% of the total colonies was randomly selected, purified and checked for catalase activity, Gram reaction and cell morphology. A total of 400 colonies were selected. Total genomic DNA was extracted from Lactobacilli overnight cultures following the method described by Gala et al. (2008). DNA concentration and DNA quality were assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). Lactobacilli isolates were subjected to genetic identification throughout multiplex-PCR, using species-specific primers targeting a conserved region of the *tuf* gene (Ventura et al., 2003). Isolates ascribed to the species *L. rhamnosus* and *L. paracasei,* were subjected to the PCR fingerprinting technique using the $(GTG)_5$ primer. Amplification was performed in a GeneAmp PCR System 9600 (Applied Biosystems) with the following temperature-time profile: 7 min 95 °C, 30 cycles of 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min, and a final step of 16 min at 65 °C. Electrophoresis was performed in 1×TAE buffer (40 mM Tris, 20 mM acetic

acid and 1 mM EDTA) on a 1.5% agarose gel (w/v) under highly standardized conditions (55 V, 400 mA, 960 min, 4 °C). Fourteen samples were loaded per gel. Four reference markers, 6 µl eachcomposed of 1.10 µlMolecular Ruler 500 bp (Bio-Rad), 1.40 µl Molecular Ruler 100 bp (Bio-Rad), 2 µl TE buffer (1mM EDTA, 10 mM Tris–HCl (pH 8.0)) and 1.50 µl loading dye, were included on every gel. Profiles were visualized under ultraviolet light after staining with ethidium bromide. Digitized images of gels were normalized and analyzed with the BioNumerics 5.1 software (Applied Maths, Belgium). Similarity matrices of densitometric curves of the gel tracks were calculated with Pearson's product-moment correlation coefficient. Cluster analyses of similarity matrices were performed by unweighted pair group method with arithmetic averages (UPGMA).To match genetic linkage the strains H25 and N24 were used as reference strains.

RESULTS

Heat resistance of probiotic strains

Probiotic lactobacilli were subjected to heat treatment (55°C for 10 minutes), that mimic condition used during Pecorino cheese production. Overall, both strains *L. rhamnosus* H25 and *L. paracasei* N24, showed high thermal resistance. Cell survival of the probiotic strain after treatment at 55°C for 10 min was similar between the H25 and N24 strains, showing a slight decrease in cell density (from 9.33±0.04 to 8.82±0.02 and from 9.14±0.02 to 8.72±0.01 log CFU/ml, respectively) (data not shown). When we induced heat adaptation of the cells at 42°C for different times (20, 30, 40, and 60 min), H25 and N24 strains exhibited an increase in cell density till 60 min, showing the greatest survival at 42°C for 30 min (from initial values of 9.33 and 9.14 to 10.08 and 9.86, respectively) (Figure 2.2). The cell density of both strains remained

quite stable till 40 min, when cells were heat adapted at 37°C of incubation, and showed a slight decrease after 60 min, registering a value of approximately 8.0 (Figure 2.2). For the promising probiotic strains H25 and N24 was, subsequently, calculated the D-value. Representative thermal death curves are illustrated in Figure 2.3. Both strains exhibited similar trend with a decrease of about 2 log cycle after treatment at 55°C for 120 min.

Figure 2.2 Survival (log_{10} CFU/g) of *L. rhamnosus* H25 and *L. paracasei* N24 strains. Cells were suspended in reconstituted skim milk at the cell density of 9.8 log_{10} CFU/ml and subjected to the following heat treatments: 55°C for 10 min after adaptation of the cells at 42°C for different times (20, 30, 40, and 60 min) (green rectangles) and after adaptation at 37°C for the same times (blue rectangles).

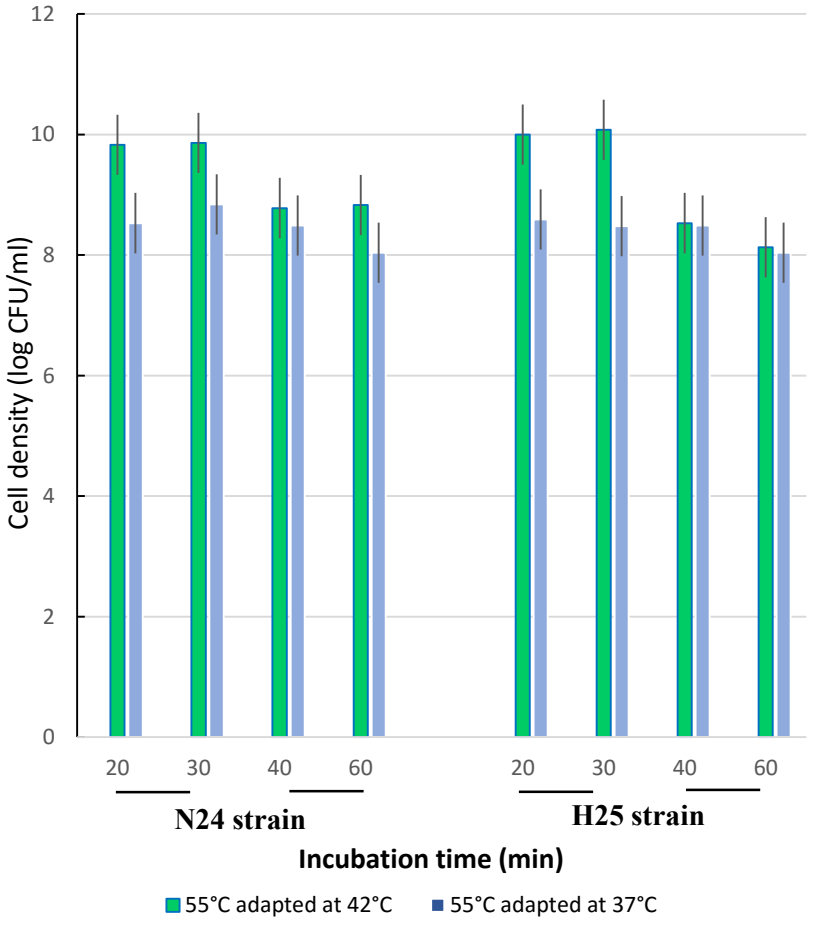
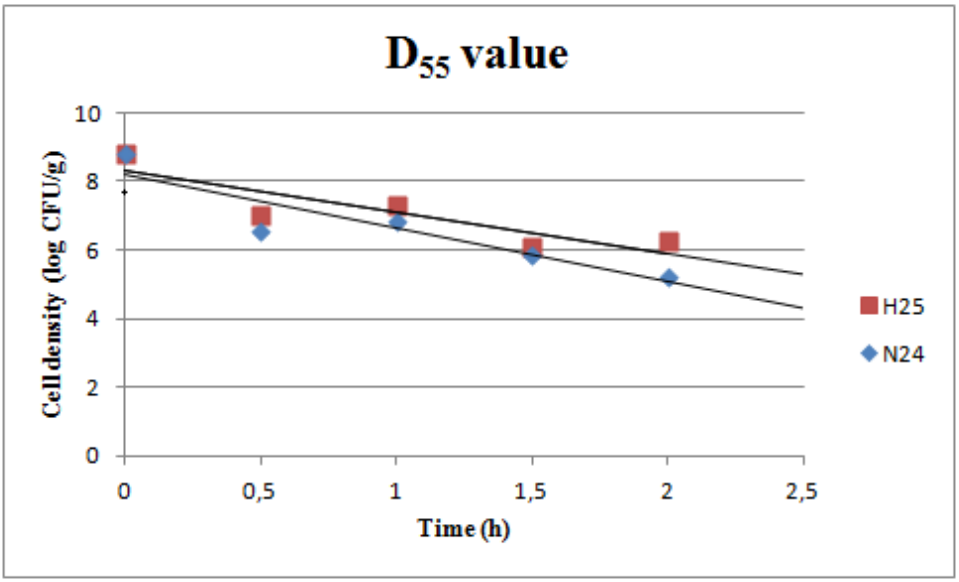


Figure 2.3 Representative rate of the decimal reduction time (D-value) of *L. rhamnosus* H25 and *L. paracasei* N24 strains. Cells were suspended in reconstituted skim milk, subjected to adaptation at 42°C for 30 min and heat treatment at 55°C for 2 hours.



Coagulant and acidifying abilities of potential probiotic strains

In Table 2.1 are reported coagulant and acidifying activities, both in skim and UHT milk, of the potential probiotic H25 and N24 strains, alone, in mixture between them and in combination with the commercial starter cultures used in the Pecorino Siciliano cheese making. Overall, when analyzed singularly, both strains exhibited good coagulant activities and good clot formation in UHT milk at 37°C for 18 h of incubation. Evaluating mixtures, the best coagulant ability and acidifying activity both in skim milk and in UHT milk were exhibited by the mixture M1 (H25 and N24 strains mixed at 1:1 ratio). The latter, showed the best coagulant and acidifying properties also when evaluated in combination with starter cultures (combination C1). For this reason, the mixture M1 was chosen to be used for the PC cheese production.

Table 2.1 Coagulant and acidifying activities of the H25 and N24 strains singly, in mixture each other and in combination with commercial starter cultures after 18h of incubation at room temperature. The coagulant and acidifying activities were also determined for the commercial starter cultures.

	Single / Mixtures	Coagulation		Δ pH	
		Skim milk	UHT milk	Skim milk	UHT milk
Single strains	**H25**	+/-	+	0.34	1.05
	N24	+/-	+	0.22	1.01
	Commercial starter cultures	+/-	++	1.04	0.99
Mixtures of strains	**Mixture M1** 1:1 (v/v) (H25&N24)	+/+	++	2.18	2.34
	Mixture M2 1:2 (v/v) (H25&N24)	+/-	+	1.03	1.15
	Mixture M3 2:1 (v/v) (H25&N24)	+/-	+	1.14	1.40
Combinations strains & starter	**Combination C1** 1:1 (v/w) (M1&starter)	+	++	0.91	1.17
	Combination C2 1:2 (v/w) (M1&starter)	+/-	+	0.80	1.00
	Combination C3 2:1 (v/w) (M1&starter)	+	++	0.81	1.07

++: very good coagulant activity; **+:** good coagulant activity; **+/-**: low coagulant activity

Microbiological analysis

Results of microbiological analysis of control (CC) and probiotic (PC) cheeses at 0, 30, 60, 90, 120 and 180 days of ripening were expressed as average of two replicates with standard deviation and were depicted in Table 2.2. Regarding control cheese (CC) the total aerobic mesophilic population showed a significant increase up to 90 days of ripening, reaching a value of 8,33 log CFU/g, followed by a decrease till 180 days of ripening (6.12 log CFU/g). Lactic acid bacteria and Lactobacilli showed the same trend, recording an increase of 1 log cycle at the end of ripening. Lactococci increased up to 90 days and significantly decreased till the 180 days of ripening. Streptococci presented trend almost constant up to 90 days, and decreased of about 2 log cycle at 120 days of ripening and after. Enterococci exhibited similar trend to those depicted from streptococci. Yeasts and *Enterobacteriaceae* revealed a constant cell density during all ripening time (average about 4 log CFU/ml).

Regarding probiotic cheese (PC), total aerobic mesophilic bacteria showed a quite constant value till 90 days of ripening, decreasing of about 2 log cycle at 180 days of ripening. Lactobacilli, as expected, achieved a high cell density at the beginning of the ripening, which significantly increased till 60 days, achieving a final value of 8.64 log CFU/ml. Lactococci were quite stable till 60 days and decreased slowly reaching the value of 7.90 CFU/ml at 180 days. Streptococci showed similar trend, achieving at the end of the ripening a significant decrease of 3 log cycle. Enterococci exhibited a constant trend till 60 days and decreased slowly, registering a final value of 7.34 CFU/ml. Yeasts showed a slight increase during ripening, recording a value of 4,84 CFU/ml at the end of ripening, while *Enterobcteriaceae* maintained a constant value for all ripening time.

Evaluating differences between probiotic and control cheeses statistical data revealed that the cheeses were significantly different only for mesophilic bacteria, LAB, and Lactobacilli groups (PCA, MRS and SL-ROGOSA media, respectively) (data not shown).

Table 2.2 Microbial counts and standard deviation (SD) of control (CC) and probiotic (PC) cheeses of the main microbial groups at different days of ripening (0-180).

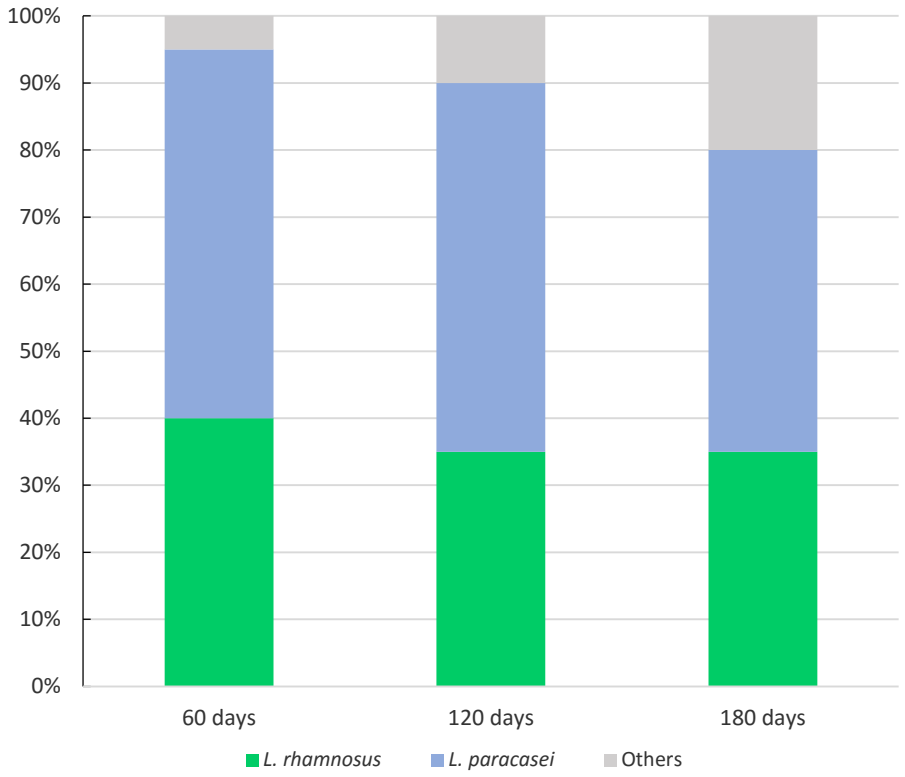
		Microbial counts (log UFC/g) and SD							
Samples	**Days**	**Mesophilic aerobic bacteria**	**LAB**	**Lactobacilli**	**Lactococci**	**Streptococci**	**Enterococci**	**Yeasts**	**Enterobacteriaceae**
CC	0	7.00±0.01^{b}	6.74±0.06^{a}	6.15±0.05^{a}	8.95±0.01^{b}	8.36±0.08^{b}	8.07±0.08^{b}	4.35±0.06^{a}	4.13±0.18^{a}
	30	8.22±0.38^{bc}	8.11±0.22^{bc}	7.33±0.14^{bc}	9.04±0.11^{b}	9.03±0.05^{d}	9.14±0.05^{d}	4.30±0.43^{a}	4.54±0.09^{a}
	60	8.33±0.04^{c}	8.19±0.27^{bc}	7.12±0.18^{bc}	8.50±0.03^{ab}	8.38±0.11^{b}	8.27±0.11^{b}	5.17±0.25^{b}	4.32±0.54^{a}
	90	8.33±0.07^{c}	8.60±0.03^{c}	7.80±0.05^{c}	9.03±0.12^{b}	8.78±0.02^{c}	8.38±0.02^{c}	3.98±0.03^{a}	3.95±0.06^{a}
	120	7.09±0.12^{b}	7.32±0.03^{b}	7.18±0.05^{b}	7.09±0.12^{a}	6.38±0.11^{a}	6.22±0.11^{a}	3.97±0.03^{a}	3.97±0.04^{a}
	180	6.12±0.05^{a}	7.78±0.00^{bc}	7.24±0.08^{bc}	7.25±0.15^{a}	6.26±0.20^{a}	6.16±0.20^{a}	4.26±0.17^{a}	3.96±0.04^{a}
PC	0	8.96±0.30^{cd}	8.97±0.04^{a}	9.02±0.06^{a}	9.11±0.15^{d}	8.86±0.02^{bc}	8.97±0.02^{c}	3.12±0.75^{a}	3.98±0.03^{a}
	30	9.11±0.18^{d}	9.37±0.09^{b}	9.11±0.08^{b}	8.87±0.03^{bc}	8.80±0.03^{bc}	8.71±0.03^{c}	3.35±0.07^{a}	4.49±0.10^{b}
	60	9.05±0.07^{cd}	9.09±0.14^{a}	9.26±0.10^{a}	9.03±0.06^{d}	9.33±0.09^{d}	9.41±0.09^{d}	3.97±0.03^{c}	3.98±0.03^{a}
	90	8.32±0.48^{bc}	8.81±0.04^{a}	8.81±0.09^{a}	8.62±0.03^{b}	8.37±0.13^{bc}	8.21±0.13^{bc}	3.69±0.02^{ab}	3.93±0.03^{a}
	120	7.06±0.68^{b}	8.95±0.07^{a}	8.54±0.05^{a}	8.69±0.01^{b}	7.00±0.01^{ab}	7.05±0.01^{a}	3.90±0.01^{bc}	3.97±0.02^{a}
	180	6.81±0.10^{a}	8.97±0.28^{a}	8.64±0.09^{a}	7.90±0.08^{a}	5.89±0.83^{a}	7.34±0.83^{ab}	4.84±0.02^{d}	3.20±0.28^{a}

Mean values of two independent samples; lowercase (a, b, etc) letters in the same column indicate different statistical significances according to Duncan test at p value of $p < 0.01$.

Viability of probiotic strains during ripening

In order to evaluate the viability of potential probiotic H25 and N24 strains in PC samples during ripening time, DNA of the colonies selected on SL-Rogosa plates at 60, 120 and 180 days of ripening were submitted to *tuf* gene multiplex PCR. As reported in Figure 2.4, *L. paracasei* N24 showed the same percentage of occurrence at 60 and 120 days of ripening with a slight decrease at 180 days of ripening. *L. rhamnosus* showed a similar percentage of occurrence in all the cheese samples analyzed.

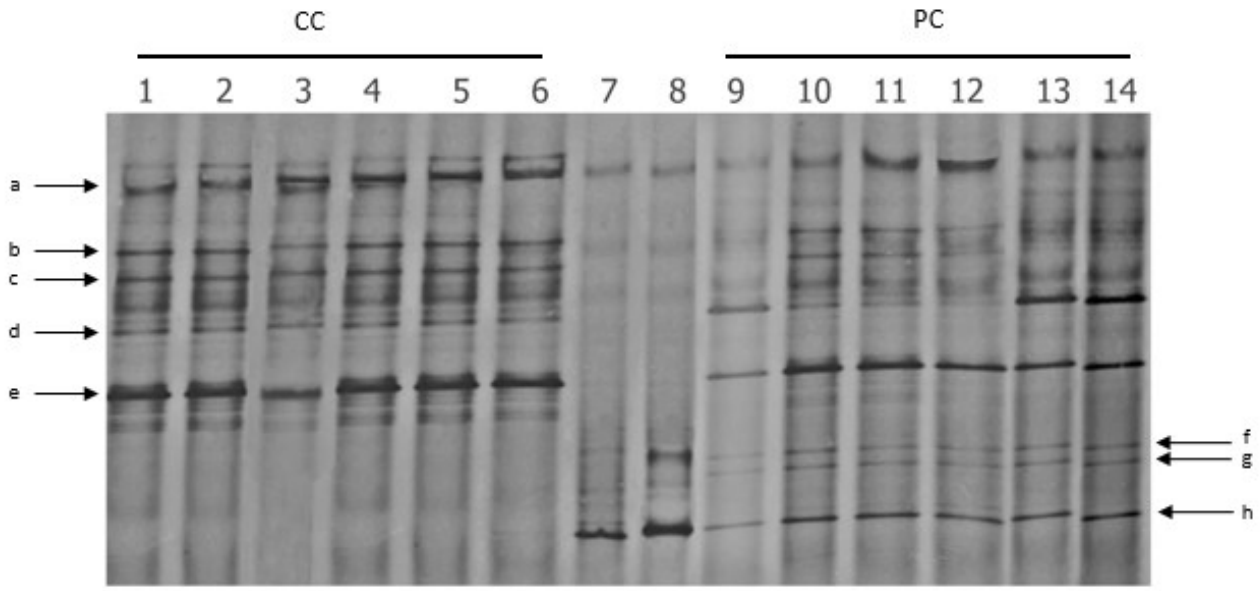
Figure 2.4 Occurrence (%) of *Lactobacillus* species in probiotic cheese (PC) at 60, 120 and 180 days of ripening.



DGGE analysis of bacterial population and identification of the dominant species

The diversity and the dynamics of the dominant microbial communities during Pecorino cheeses ripening without and with the addition of probiotic strains (CC and PC respectively), were analyzed by PCR-DGGE using primers HDA1 and HDA2, targeting the V2-V3 region of 16S rDNA. Results are shown in Figure 2.5. The appearance and disappearance of amplicons in the DGGE patterns indicate important shifts in the microbial community structure. Overall, microbial community of both CC and PC cheeses were quite stable during the ripening (0-180 days) with several bands in common. Comparison of profiles generated from *L. rhamnosus* H25 (line 7) and *L. paracasei* N24 (line 8) with those generated from CC and PC cheese samples, showed the presence of both species only in PC samples till 180 days of ripening. Clone libraries of the 16S rDNA amplicons from the 60, 120 and 180 days old CC and PC samples were constructed in order to identify some of the dominant bands in the rDNA-derived patterns. In detail, the dominant band *a*, which corresponded to *Streptococcus macedonicus* species, the dominant bands *b*, *c* and *d* corresponded to *Streptococcus termophilus* species and the band *e* corresponded to *Lactococcus lactis* were detected in all samples investigated and throughout the ripening. In addition, in the PC cheese was recorded the dominance of *Lactoacillus casei/rhamnosus* species (bands *f* and *h*) and of *Lactobacillus fermentum* (band g) during the ripening time.

Figure 2.5 DGGE profiles of the V2-V3 16S rRNA gene of control and probiotic samples at 0, 30, 60, 90, 120 and 180 days of ripening.



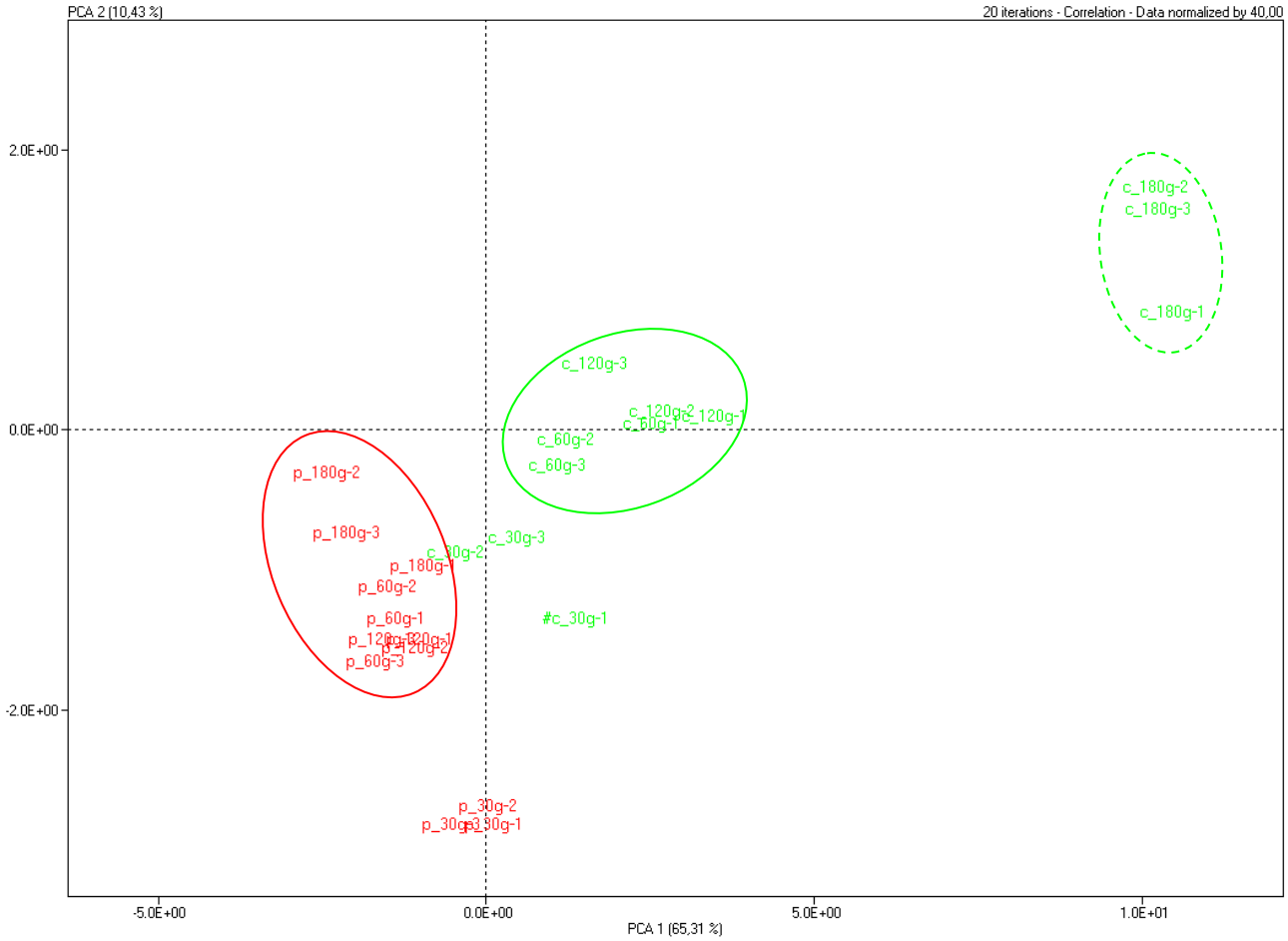
Lines 1-6, control cheese (CC) at 0, 30, 60, 90, 120, and 180 days of ripening respectively; line 7, *L. rhamnosus* H25 strain; line 8, *L. paracasei* N24; lines 9-14, probiotic cheese (PC) at 0, 30, 60, 90, 120, and 180 days of ripening respectively. Band a, *Streptococcus macedonicus;* bands b, c, and d, *Streptococcus termophilus;* band e, *Lactococcus lactis*; bands f and h, *Lactoacillus casei/rhamnosus;* band g, *Lactobacillus fermentum*.

Smart Nose analysis

All data sets from SMart Nose results were gathered using the software SMart Nose 1.51. The PCA obtained by SMart Nose analysis of PC and CC samples is shown in Figure 2.6. The PCA two-dimensional plots shows the data obtained for the analysis of probiotic (PC) and control (CC) cheeses at 30, 60, 120, and 180 days of ripening. The volatile profile of Pecorino cheese was mainly affected by the different strains used. The CC and the PC samples showed a good

separation with PC1 (65.31%) and PC2 (10.43%). In detail, CC samples at 180 days of ripening (in green dotted line) displayed a further separation from the others at days 60 and 120 (green line). Probiotic samples in red group showed a higher variability for samples at 30 days that were slightly distant, but not clearly separated, from the samples at 60-120 and 180 days (red line).

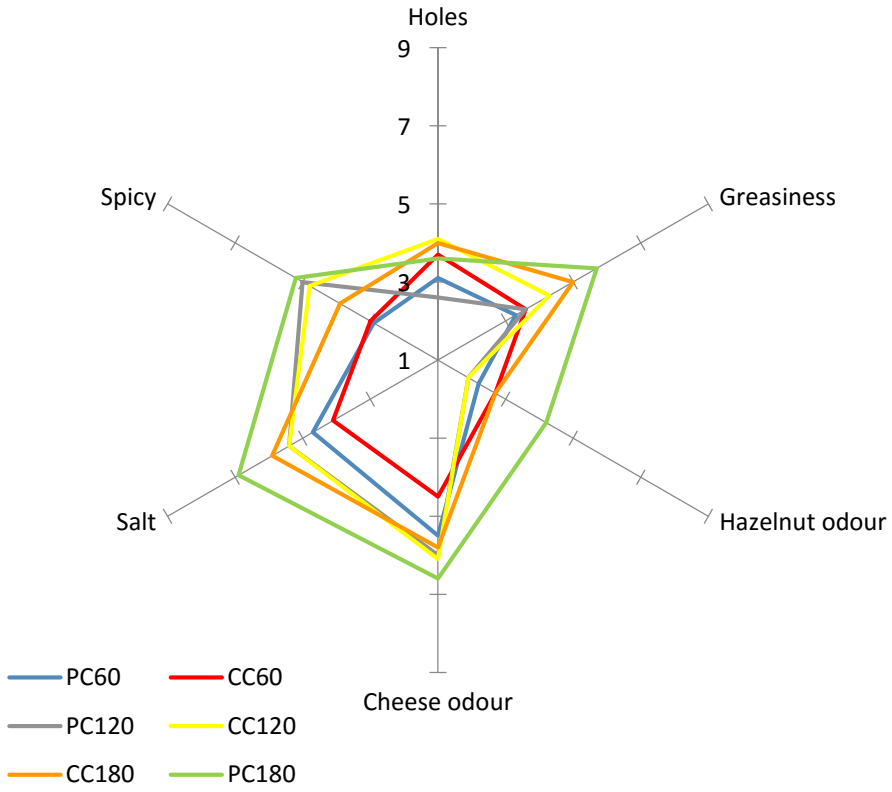
Figure 2.6 PCA two-dimensional plots of SMart Nose analysis of probiotic PC (green) and control CC (red) samples at 30, 60, 120 and 180 days of ripening.



Sensory analysis

ANOVA results of sensory analysis showed that PC and CC cheeses at 60, 120 and 180 days of ripening were significantly different ($p < 0.05$) for six of the twenty descriptors selected (figure 2.7). In detail, the highest value for the descriptor holes was attributed to CC at 120 and 180 days of ripening while, salt and greasiness were valuated more intense in both PC and CC samples 180 days old. PC obtained the highest value for spicy (120 and 180 days of ripening) and hazelnut and cheese odours (180 days old). Results of triangle test are illustrated in Table 2.3. In detail, a mean of 60% of correct answers was attributed to cheeses at 120 and 180 days of ripening, while 77% of correct answers were obtained for cheese at 90 days of ripening. From data clearly appears that the panellists were able to differentiate significantly the CC and PC samples at each ripening time considered in the present study. In addition, as reported in table 2.4, PC cheese obtained a significant preference at 90 days of ripening while the panellists showed the preference for CC cheese at 120 and 180 days of ripening.

Figure 2.7 Sensory profile of probiotic (PC) and control (CC) cheeses at 60, 120 and 180 days of ripening



		CC			PC		
	Descriptors	**60**	**120**	**180**	**60**	**120**	**180**
	Colour	4.7	5.1	4.9	3.9	4.8	4.9
	Holes	3.7^{ab}	4.1^{b}	4.0^{b}	3.1^{ab}	2.6^{a}	3.6^{ab}
	Greasiness	3.6^{a}	4.3^{ab}	5.0^{bc}	3.3^{a}	3.6^{a}	5.7^{c}
	Compactness	5.8	5.9	6.1	5.3	5.2	5.7
	Salt	4.1^{a}	5.4^{abc}	5.9^{bc}	4.7^{ab}	5.4^{abc}	6.9^{c}
	Acid	2.3	2.9	3.0	3.6	3.3	3.2
	Bitter	1.5	2.7	1.9	2.0	2.9	2.7
	Spicy	3.0^{ab}	4.8^{bc}	3.9^{abc}	2.9^{a}	5.0^{c}	5.2^{c}
Odour	Milk	4.1	4.0	3.8	5.0	4.3	3.9
Odour	Butter	3.4	4.4	4.7	3.7	4.4	4.1
Odour	Fruits	1.8	2.1	3.3	1.7	2.8	2.9
Odour	Hazelnut	2.7^{ab}	1.9^{a}	2.7^{ab}	2.2^{a}	1.9^{a}	4.2^{b}
Odour	Cheese	4.5^{a}	6.1^{ab}	5.8^{ab}	5.5^{ab}	6.0^{ab}	6.6^{b}
Odour	Off-odour	1.4	1.8	1.8	1.4	1.7	1.6
Taste	Milk	4.7	4.2	5.0	4.0	4.3	4.3
Taste	Butter	3.6	3.6	4.1	3.8	4.2	3.8
Taste	Fruits	1.8	2.3	2.8	1.8	2.6	2.6
Taste	Hazelnut	1.9	1.9	3.2	2.6	2.1	3.3
Taste	Cheese	5.2	6.0	5.9	5.6	6.3	5.8
Taste	Off-flavour	1.3	1.4	1.6	1.6	1.7	2.3

Mean value attributed to each descriptor. Lowercase (a, b, etc) letters in the same column indicate different statistical significances ($p<0.05$)

Table 2.3 Statistical difference between control (CC) and probiotic (PC) Pecorino cheese samples obtained by triangle test.

Days of Ripening	Comparison	Correct Answers	% of Correct Answers	Significant difference α=0.05
90-days	CC vs PC	23	77%	*
120-days	CC vs PC	18	60%	*
180-days	CC vs PC	19	63%	*

* 15 is the minimum number of correct responses needed to conclude that a perceptible difference exists based on a triangle test were N=30; α=0.05; β=0.10 and pd=40%.

Table 2.4 Statistical difference between control (CC) and probiotic (PC) Pecorino cheese samples based on preference test.

Days of Ripening	Comparison	Correct Answers	CC Preference	PC Preference
90-days	CC vs PC	23	8	15
120-days	CC vs PC	18	10	8
180-days	CC vs PC	19	14†	5

† Significant difference on unilateral binomial test π=0.50; α=0.05.

Faecal samples analysis

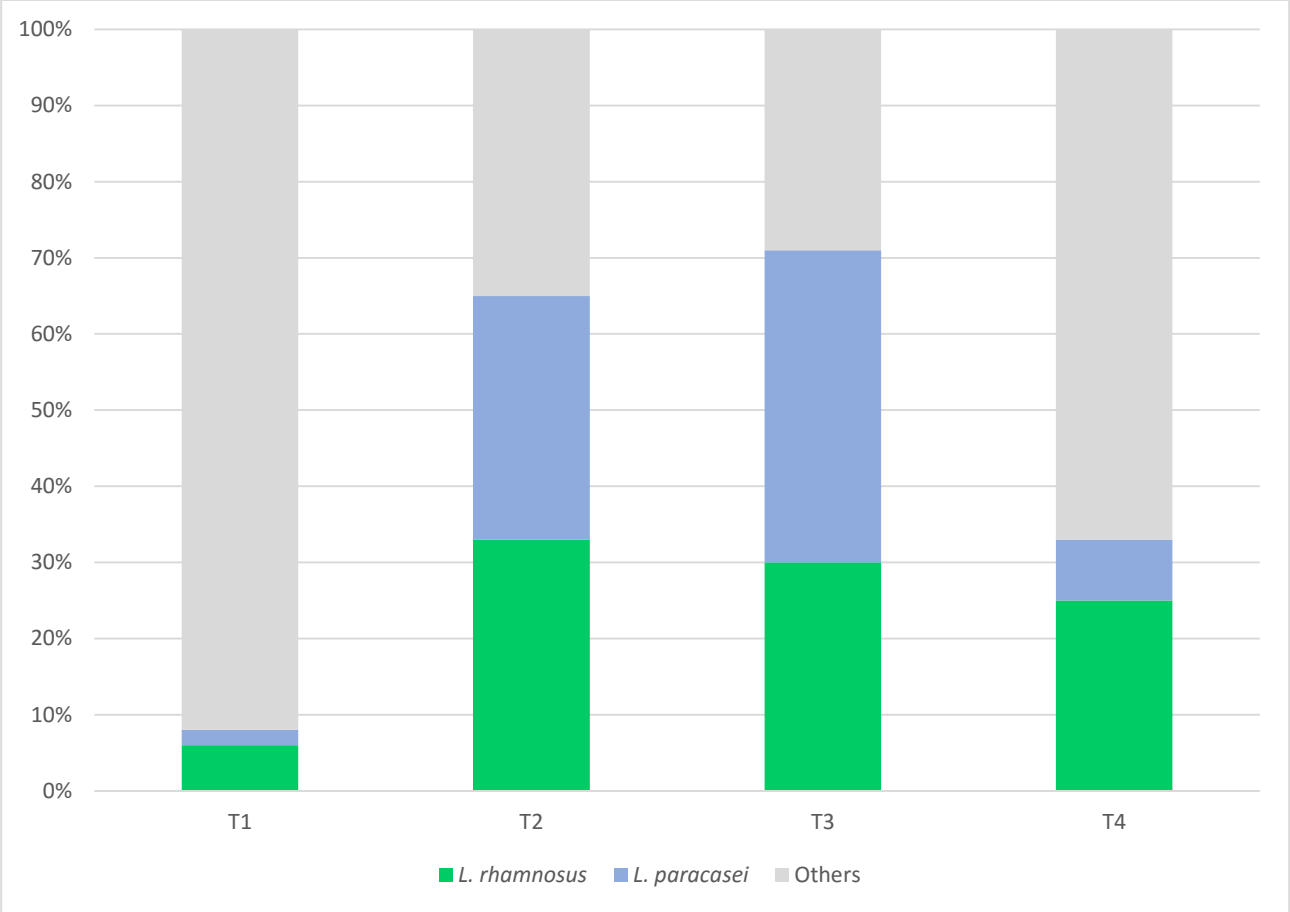
The mean of microbial counts and standard deviation obtained by enumeration of lactobacilli population present in faecal samples of volunteers participated to the study, collected during each sampling time (T1, T2, T3, and T4), are shown in Table 2.5. The cellular density detected at baseline sampling time (T1) was variable among patients with a mean value of 4.97 log CFU/g of faeces. The highest lactobacilli value was achieved by volunteer B (7.47 log CFU/g) while, the lowest by volunteers C and L (3.30 log CFU/g and 3.04 log CFU/g respectively). During the probiotic cheese assumption both for 7 (T2) and 15 (T3) days, the *Lactobacillus* population increased in all patients with the exception for the B volunteer, which showed at T2 sampling time a slight decrease. When the probiotic cheese administration was stopped (T4, 2 weeks after the end of the assumption) the *Lactobacillus* population was subjected to a slight decrease in cell density in all volunteers except for L one which registered an increase of 1 log unit.

In order to study the *Lactobacillus* population a total of 400 colonies, 10 for each sampling time and each volunteers, were isolated from SL-Rogosa plates and subjected to *tuf* gene PCR. The occurrence (%) of *L*. *rhamnosus* and *L*. *paracasei* species are reported in figure 2.8. In detail, *L*. *rhamnosus* and *L*. *paracasei* represented at baseline (T1) the 6% and 2% of the total isolates respectively. These percentages of distribution increased sensibly after 7 days of probiotic cheese intake (T2) shifted to 33% for *L*. *rhamnosus* and 32% for *L*. *paracasei* and remained quite stable at T3 sampling time (30% *L*. *rhamnosus* and 41% *L*. *paracasei*). During the 2-weeks follow-up period the occurrence of *L*. *rhamnosus* and *L*. *paracasei* decreased to 25% and 8% respectively.

Table 2.5 *Lactobacillus* population counts, in SL-Rogosa medium, and standard deviation (SD) of faecal samples of 10 subjects (A-L) collected during the trial at baseline (T1), 1-weeks probiotic cheese administration (T2), 2- weeks probiotic cheese administration (T3), 2-weeks follow up period (T4).

Subjects	Sampling time			
	T1	**T2**	**T3**	**T4**
A	4.65±0.04	5.20±0.05	7.34±0.04	6.60±0.02
B	7.47±0.05	7.28±0.04	8.30±0.03	7.43±0.02
C	3.30±0.03	4.30±0.05	7.07±0.03	5.51±0.03
D	5.04±0.03	5.68±0.02	7.64±0.04	5.90±0.05
E	5.92±0.06	6.69±0.02	8.74±0.05	7.14±0.05
F	4.32±0.04	5.00±0.07	6.30±0.02	5.85±0.04
G	5.64±0.04	6.93±0.05	7.91±0.03	7.07±0.03
H	5.50±0.03	5.90±0.03	7.72±0.03	6.20±0.02
I	4.91±0.02	5.49±0.03	6.67±0.04	5.75±0.03
L	3.04±0.02	4.60±0.03	5.88±0.03	6.70±0.02

Figure 2.8 Occurrence (%) of *L. rahmnosus* and *L. paracasei* strains isolated from faecal samples of 10 volunteers at baseline (T1), 1-weeks probiotic cheese administration (T2), 2-weeks probiotic cheese administration (T3), 2-weeks follow up period (T4).



Genetic identification of *L. rhamnosus* H25 and *L. paracasei* N24 strains

(GTG)5-PCR fingerprints were generated from 98 isolates (88 derived from faecal samples and 10 isolated from the cheese administrated during the trial) and cluster analysis was performed (figure figure 2.9). Moreover, a dendogram with isolates that clustered with the reference strains (H25 and N24) were also constructed (figure 2.10). Concerning the isolates originated

form probiotic cheese, 2 isolates clustered with H25 strain and 4 clustered with N24 strain. Among the strains isolated from faecal samples, 30 isolates clustered with H25 and 21 clustered with N24 strains. Thirty-seven isolated did not showed similar GTG_5 profile to the reference strains. Zooming on *L. rhamnosus* strains closely related to *L. rhamnosus* H25 strain, 13 derived from faecal samples at 7 days of cheese administration (T2), 16 derived from 15 days administration (T3) and only one from 2 weeks after the end of cheese assumption (T4). Similarly, the majority of the strains that clustered with N24 derived from 15 days of cheese administration (13 isolates).

Figure 2.9 Dendrogram generated after cluster analysis of the digitized (GTG)5-PCR fingerprints of the Lactobacilli (98) isolated from cheese and faecal samples.

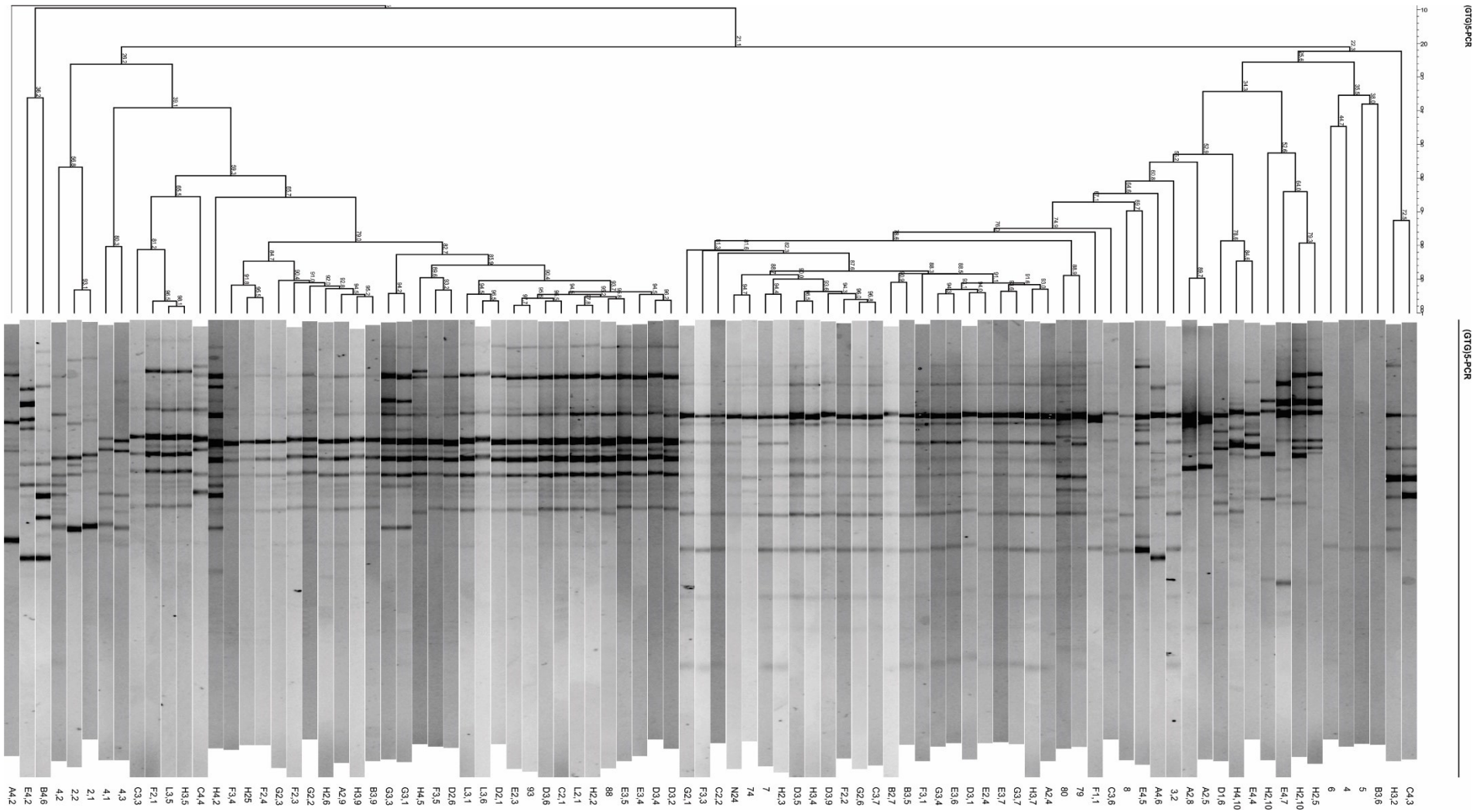
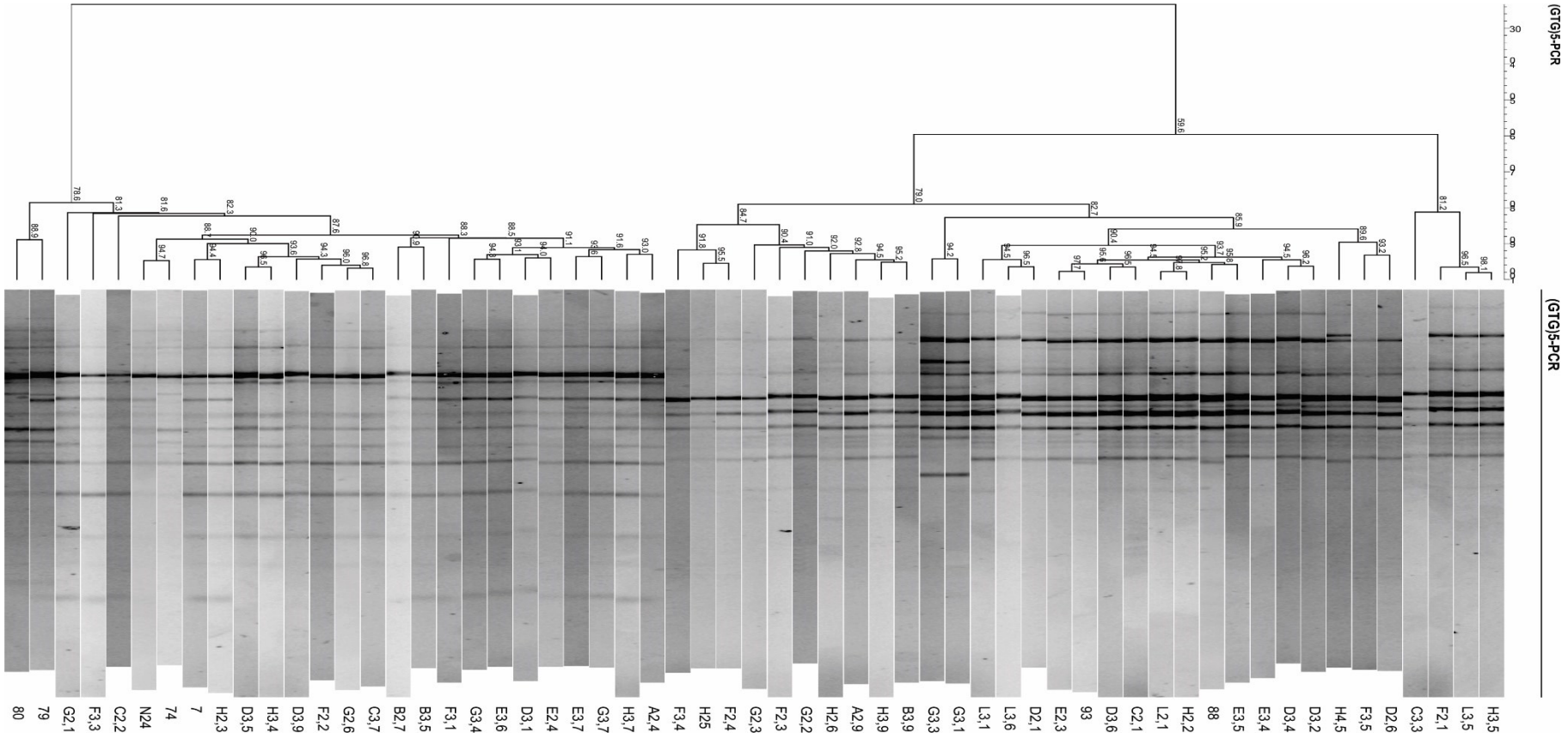


Figure 2.10 Dendrogram generated after cluster analysis of the digitized (GTG)5-PCR fingerprints of the Lactobacilli isolated from cheese (6) and faecal (51) samples that exhibited identical profile to H25 and N24 reference strains.



DISCUSSION

In the present study two promising probiotic strains *Lactobacillus rhamnosus* H25 and *Lactobacillus casei* N24 were used to produce experimental probiotic Pecorino Siciliano cheese and their impact during the ripening period was investigated. Both strains exhibited good technological properties in terms of coagulant and acidifying abilities both in skim and UHT milk. It is interesting to point out that when the cells were combined with commercial starter cultures the coagulant activity was higher. This could be due to a possible positive interaction among the promising strains and the starter cultures generally used for Pecorino Siciliano cheese making. This evidence was supported by the microbial counts and the DGGE results of the V2-V3 regions of the 16S rRNA gene. Microbial data showed an increase of lactococci and streptococci during the ripening, especially from 90 till 180 days, confirmed by DGGE profiles which revealed the dominance of *Lactococcus lactis* and *Streptococcus thermophilus* in both control and probiotic cheeses. These results are in contrast to Diezhandino et al., 2015 (12), which observed a decrease of lactococci during ripening of Valdeón cheese, a traditional blue cheese produced in Spain. The occurrence of enterococci at high level highlights their contribution during the cheese ripening, as already suggested by Suzzi and co-workers (2000) (44). The higher level of lactobacilli at the beginning of ripening in probiotic cheese respect to the control one could be explained by their ability to survive during heat treatment (55°C for 10 min). This result is in contrast to those obtained by Cárdenas et al., (2014) (5) which demonstrated a loss in probiotic strains when they were co-cultured with starters. In addition the viability of the H25 and N24 strains was improved (almost 1 log unit) when cells were adapted at 42 °C for 30 min, in accordance with previous studies (9, 25). The strains showed high resistance to heat conditions that mimic curd cooking

generally used for making Pecorino Siciliano cheese. Even the Pecorino cheese is made from pasteurized milk and the curd is cooked at 55°C the *Enterobacteriaceae* level was quite high both in probiotic and control cheeses. Similar finding was achieved in Mozzarella cheese and Fior di latte cheese (10, 42, 25). In order to have industrial application, is widely accepted that the incorporated probiotic strains must maintain their viability during the production, throughout the shelf life of the product and up to the time of the consumption. In the present study, the LAB and lactobacilli cell densities significantly increased during the ripening time and both *L. rhamnosus* H25 *and L. paracasei* N24 strains remained viable in the probiotic cheese until 180 days of ripening.

Cheese has been considered as an excellent alternative to fermented milk and yogurts as food vehicle for probiotic delivery (50, 51, 27, 28, 3, 4). The buffering capacity is one of its advantages because it protects probiotics against the highly acidic stomach environment. The structure of the gel and its high fat content and solid consistency also add to the probiotic protection (27, 28). However, variable results have been obtained with different probiotic strains and each strain should be tested individually. In the present study, in order to detect the two promising strains (*Lactobacillus rhamnosus* H25 and *Lactobacillus casei* N24) in the probiotic cheese at the end of the ripening time (180 days old), the GTG_5-PCR analysis was carried out. Moreover, the same technique was applied to reveal the strains survival in fecal samples of 10 adult volunteers, which consumed daily the probiotic cheese for 15 days. Our results demonstrated that the promising probiotic strains were found in cheese and exhibited excellent viability during the gastrointestinal tract transit. In fact, the cell density of total lactobacilli increased in fecal samples during the trial. It is well established that the ingestion of certain probiotic may increase the total number of indigenous lactobacilli (40, 19, 52, 36,

37). Microbiota changes, attributable to probiotic intake, include increase numbers of related phylotypes and decrease in pathogens and their toxins (36, 37). The consumption of probiotic cheese did not cause abdominal pain, flatulence, bloating or their combination in volunteers. Nevertheless, few volunteers reported constipation after 2 weeks of cheese ingestion. It is well known that cheese is rich in saturated fats ad proteins and lacks fiber hence, an excessive consumption of cheese could result in constipation (37). Actually, consumers demand the addition of probiotic cultures to many foods, including cheese, but a primary consideration is that the sensory properties, especially taste, should be appealing. Although according to the preference test panellists exhibited a significant preference of PC cheese at 90 days of ripening and the sensorial profiles revealed differences between probiotic and control cheeses only for six out of the twenty descriptors selected. It is interesting to highlight that the addition of probiotic strains did not affect the bitterness level in cheese. This could be due to the ability of the strains to degrade bitter peptides, as reported by Martinez-Cuesta et al 2001 (23).

In conclusion, the results of the present study point out the excellent technological properties of the promising probiotic *Lactobacillus rhamnosus* H25 and *L. paracasei* N24 strains, suggesting their possible use during the manufacture of the ripened Pecorino Siciliano cheese. In addition, we demonstrated that both strains survived throughout cheese ripening, indicating that aged-cheese is a suitable vehicle for probiotic delivery in the gastro intestinal tract. Further, we also demonstrated their viability in human faeces, suggesting their possible role on the metabolism and composition of GI microbiota.

REFERENCES

1. Bergamini C.V., Hynes E.R., Quiberoni A., Suárez V.B., Zalazar C.A. (2005). Probiotic bacteria as adjunct starters: influence of the addition methodology on their survival in a semi-hard Argentinean cheese. Food Research International, 38, 597–604.
2. Bhadoria P.B.S., Mahapatra S.C. (2011). Prospects, Technological Aspects and Limitations of Probiotics – A Worldwide Review. European Journal of Food Research & Review, 1, 23-42.
3. Buriti F.C.A., da Rocha J.S., Saad S.M.I. (2005). Incorporation of *Lactobacillus acidophilus* in Mina fresh cheese and its implications for textural and sensorial properties during storage. International Dairy Journal, 15, 1279-1288.
4. Buriti F.C.A., da Rocha J.S., Assis G., Saad S.M.I. (2005). Probiotic potential of Mina fresh cheese prepared with the addition of *Lactobacillus paracasei*. Food Science and Technology, 38, 173-180.
5. Cárdenas N., Calzada J., Peirotén A., Jiménez E., Escudero R., Rodríguez J.M., Medina M., Fernández L. (2014). Development of a potential probiotic fresh cheese using two *Lactobacillus salivarius* strains isolated from human milk. Hindawi Publishing Corporation, 2014, 1-12.
6. Carminati D., Perrone A., Neviani E. (2001). Inhibition of *Clostridium sporogenes* growth in Mascarpone cheese by co-inoculation with *Streptococcus thermophilus* under conditions of temperature abuse. Food Microbiology, 18, 571–579.
7. Carpino S., Rapisarda T., Belvedere G., Licitra G. (2008). Volatile fingerprint of Piacentinu cheese produced with different tools and type of saffron. Small Ruminant Research, 79, 16–21.
8. Castro J.M., Tornadijo M.E., Fresno J.M., Sandoval H. (2015). Biocheese: A Food Probiotic Carrier. BioMed Research International, 2015, Article ID 723056.
9. De Angelis M., Gobbetti M. (2004). Environmental stress responses in *Lactobacillus*; a review. Proteomics, 4, 106-122.
10. De Angelis M., de Candida S., Calasso M.P., Faccia M., Guinee P., Simonetti M.C., Gobbetti M. (2008). Selection and use of autochthonous multiple strain cultures for

the manufacture of high-moisture traditional Mozzarella cheese. International Journal of Food Microbiology, 125, 123-132.

11. De Souza C.H.B., Buriti F.C.A., Behrens J.H., Saad S.M.I. (2008). Sensory evaluation of probiotic Minas fresh cheese with *Lactobacillus acidophilus* added solely or in co-culture with a thermophilic starter culture. International Journal of Food Science and Technology, 43, 871–877.

12. Diezhandino I., Fernández D., González L., McSweeney P.L.H., Fresno J.M. (2015). Microbiological, physic-chemical and proteolytic changes in Spanish blue cheese during ripening (Veldeón cheese). Food Chemistry, 168, 134-141.

13. Doyon M., Labrecque J.A. (2008). Functional foods: a conceptual definition. British Food Journal, 11, 1133-1149.

14. Food and Agriculture Organization of the United Nations, World Health Organization—FAO/WHO. 2001. Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Spain: Food and Agriculture Organization of the United ´ Nations, World Health Organization.

15. Fritzen-Freire C.B., Muller C.M.O., Laurindo J.O.B., Amboni R.D.M.C., Prudencio E.S. (2010). The effect of direct acidification on the microbiological, physicochemical and sensory properties of probiotic Minas Fresch cheese. International Journal of Dairy Technology, 63, 561–568.

16. Gala, E., Landi, S., Solieri, L., Nocetti, M., Pulvirenti, A., Giudici, P. (2008). Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. International Journal of Food Microbiology, 125, 347-351.

17. Gobbetti M., Corsetti A., Smacchi E., Zocchetti A., de Angelis M. (1997). Production of Crescenza cheese by incorporation of bifidobacteria. Journal of Dairy Science, 81, 37–47.

18. Gomes da Cruz A., Buriti F.C.A., Batista de Souza H., Fonseca Farina A., Isay Saad S.M. (2009). Probiotic cheese health benefits, technological and stability aspects. Trends in Food Science and Technology, 20, 344-354.

19. Goossens D., Jonkers D., Russel M., Stobberingh A., Van Den Bogaard A., stockbrügger R. (2003). The effect of *Lactobacillus plantarum* 299v on the bacterial composition and metabolic activity in faeces of healthy volunteers; a placebo controlled study on the

onset and duration of effects. Alimentary Pharmacology and Therapeutics, 18, 495-505.

20. Hayes M., Coakley M., O'Sullivan L., Stanton C., Hill C., Fitzgerald G.F., Murphy J.J., Ross R.P. (2006). Cheese as a delivery vehicle for probiotics and biogenic substances. Australian Journal of Dairy Technology, 61, 132–141.

21. Heller K.J., Bockelmann W., Schrezenmeir J., deVrese M. (2003). Cheese and its potential as a probiotic food. Handbook of fermented functional foods, 203–225.

22. Karimi R., Mortazavian A.M., Gomes Da Cruz A. (2011). Viability of probiotic microorganisms in cheese during production and storage: a review. Dairy Science & Technology, 91, 283-308.

23. Martínez-Cuesta M.C., Fernández de Palencia P., Requena T., Peláez T (2001). Enzymatic ability of *Lactobacillus casei* subsp. *casei* IFPL731 for flavour development in cheese. International Dairy Journal, 11, 577-585.

24. Masuda T., Yamanari R., Itoh T. (2005). The trial for production of fresh cheese incorporated probiotic *Lactobacillus acidophilus* group lactic acid bacteria. Milchwissenschaft, 60, 167–71.

25. Minervini F., Siragusa S., Faccia M., Dal Bello F., Gobbetti M., De Angelis M. (2012). Manufacture of Fior di Latte cheese by incorporation of probiotic lactobacilli. Journal of Dairy Science, 95, 508–520.

26. Muyzer G., Small K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek, 73, 127-141.

27. Ong L., Henriksson A., Shah N.P. (2006). Development of probiotic Cheddar cheese containing *Lb. acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal, 16, 446–456

28. Ong L., Henriksson A., Shah N.P. (2007). Chemical analysis and sensory evaluation of Cheddar cheese produced with *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* or *Bifidobacterium* sp. International Dairy Journal, 17, 937–945.

29. Pitino I., Faulks R.M., Randazzo C.L., Le Marc Y., Mandalari G., Lo Curto A., Bisignano C., Caggia C., Wickham M.S.J. (2010). Survival of *Lactobacillus rhamnosus* strains in the upper gastrointestinal tract. Food Microbiology, 27, 1121-1127.

30. Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M., Vaughan, E.E. (2002). Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied Environmental Microbiology, 68, 1882-1892.

31. Randazzo C.L., Pitino I., Ribbera A., Caggia C. (2010). Pecorino Crotonese cheese: Study of bacterial population and flavour compounds. Food Microbiology, 27, 363-374.

32. Randazzo C.L., Pitino I., Caggia C. (2009). Sicurezza di ceppi probiotici. Ricerca.

33. Rerfroid MB. (2000). Concepts and strategy of functional food science: the European perspective. American Journal of Clinical Nutrition, 71, 1660–1664.

34. Roberfroid M.B. (2002). Functional foods: concepts and application to inulin and oligofructose. British Journal of Nutrition, 87, 139-143.

35. Ross R.P., Fitzgerald G., Collins K., Stanton C. (2002). Cheese delivering biocultures: probiotic cheese. Australian Journal of Dairy Technology, 57, 71–78.

36. Sangisepp E., Mikelsaar M., Rätsep M., Zilmer M., Hütt P., Utt M., Zilmer K., Uksti J., Kõljalg S. (2009). Isolated microorganism *Lactobacillus plantarum* Tensia DSM 21380 as antimicrobial and antihypertensive probiotic, food product and composition comprising said microorganism and use of said microorganism for preparation of antihypertensive medicine and method for suppressing pathogens and non-starter lactobacilli in food. Bio-Competence Centre of Healthy Dairy Products LLC, assignee. Pat. Appl. N WO2009138091.

37. Sangisepp E., Hütt P., Rätsep M., Shkut E., Kõljalg S., Truusalu K., Stsepetova J., Smidt I. Kolk H., Zagura M., Mikelsaart M. (2012). Safety of a probiotic cheese containing *Lactobacillus plantarum* Tensia according to a variety of health indices in different age groups. Journal of Dairy Science, 95, 5495-5509.

38. Sanguinetti C.J., Dias Neto E., Simpson A.J. (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechnology. 17, 914-921.

39. Saxelin M., Korpela R., Maÿrä-Mäkinen A. (2003). Introduction: classifying functional dairy products. Functional Dairy Products, 1–16.

40. Sepp E., Mikelsaar M., Salminen S. (1993). Effects of administration of lactobacillus casei stain GG on the gastrointestinal microbiota in new-borns. Microbial Ecology in Health and Diseases, 6, 309-314.

41. Sharp M.D., McMahon D.J., Broadbent J.R. (2008). Comparative evaluation of yogurt and low-fat Cheddar cheese as delivery media for probiotic *Lactobacillus casei*. Journal of Food Science, 73, 375–377.

42. Sinigaglia M., Bevilacqua A., Corbo M.R., Pati S., Del Nobile M.A. (2008). Use of active compounds for prolonging the shelf life of mozzarella cheese. International Dairy Journal, 18, 624-630.

43. Souza C.H.B., Buriti F.C.A., Behrens J.H., Saad S.M.I. (2008). Sensory evaluation of probiotic Minas fresh cheese with *Lactobacillus acidophilus* added solely or in co-culture with a thermophilic starter culture. International Journal of Food Science and Technology, 43, 871–877.

44. Suzzi G., Caruso M., Gardini F., Lombardi A., Vannini L., Guerzoni M. (2000). A survey of the Enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). Journal of applied microbiology, 89, 267-274.

45. UNI 10957 (2003). Sensory analysis—Method for establishing a sensory profile in foodstuffs and beverages. Milano, Italia: UNI: Ente Nazionale Italiano di Unificazione.

46. UNI ISO 4120 (2004) Sensory analysis - Methodology for Triangle test. Milano, Italia: UNI: Ente Nazionale Italiano di Unificazione.

47. UNI ISO 5495 (2005) Sensory analysis - Methodology- Paired comparison test. Milano, Italia: UNI: Ente Nazionale Italiano di Unificazione.

48. Ventura M., Canchaya C., Meylan V., Klaenhammer T.R., Zink R. (2003). Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. Applied and Environmental Microbiology, 69, 6908-6922.

49. Verschuren P.M. (2002). Functional Foods: Scientific and Global Perspectives. British Journal of Nutrition, 88, 125–130.

50. Vinderola C.G., Prosello W., Ghiberto D., Reinheimer J.A. (2000). Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and non-probiotic microflora in Argentinian Fresco cheese. Journal of Dairy Science, 83, 1905–1911.

51. Vinderola C.G., Prosello W., Molinari F., Ghiberto D., Reinheimer J.A. (2009). Growth of *Lactobacillus paracasei* A13 in Argentinian probiotic cheese and its impact on the characteristics of the product. International Journal of Food Microbiology, 135, 171–174.

52. Wind R.D., Tolboom H., Klare I., Huys G., Knol J. (2010). Tolerance and safety of the potentially probiotic strain Lactobacillus rhamnosus PRSF-L477: a randomized double-blind placebo controlled trial in healthy volunteers. British Journal of Nutrition.

Chapter 3

Lactobacillus rhamnosus in table olives production

Giarraffa and Grossa di Spagna naturally fermented table olives: Effect of starter and probiotic cultures on chemical, microbiological and sensory traits

C.L. Randazzo, A. Todaro, A. Pino, I. Pitino, O. Corona, A. Mazzaglia, C. Caggia

Food Research International 62 (2014) 1154–1164

Giarraffa and Grossa di Spagna naturally fermented table olives: Effect of starter and probiotic cultures on chemical, microbiological and sensory traits

INTRODUCTION

Table olives are the most important fermented vegetables on the international food market, with an estimated production of 2,563,000 tons per year (22). The European Union (EU) is the main producing area in the world and Spain is the leading producing country, followed by Greece and Italy, with the 15.2% and 9.6% of the table olives marketed in the EU, respectively. Several ways are followed to produce table olives, and the most widespread is the Spanish style method. In Italy, 44% of the table olives are yielded in Sicily and the pro capita yearly consumption is about 3 kg, one-third of the domestic production (51). In Eastern Sicily, the manufacturing process of table olives is mainly performed at the olive farming level. After picking, the green olives are directly placed under brine, with an initial salt concentration of 8–10% (38). The drupes in the brine undergo a mixed-acid fermentation until, at least partially, they lose their bitterness. The fermentation is a non-controlled process, inwhich cultivars, physico-chemical conditions (salt content and temperature) as well as indigenous microbiota of olives influence the quality of the final product (5, 30, 47). In particular, un-debittered green table olives represent a complex habitat in which indigenous lactic acid bacteria (LAB) and yeasts co-interact (5, 28, 39). The synergism between LAB and yeasts is of fundamental importance in obtaining high quality products (20). LAB rapidly metabolize the sugar available producing lactic acid, which originates the rapid acidification of brines (16) while yeasts contribute to enhance LAB growth releasing nutritive compounds (5). Both microbial groups are involved in the volatile compound and metabolite production from major olive

constituents, through various biochemical pathways. Alcohols, esters, aldehydes and ketones, as well as acids, are known to be formed by metabolic activities of LAB and yeasts, which improve the flavor properties of final product (25). Nevertheless, spontaneous fermentation is often unpredictable and an excessive growth of yeast usually determines a final product with a milder taste and less self-preservation due to the high pH (30). Thus, suitable starter cultures could be used in order to standardize the process and to obtain replicable, high quality and safe products. Several LAB strains and mixed cultures of yeasts and LAB have been developed as starters or protective cultures and some commercial starter cultures of *Lactobacillus pentosus* or *L. plantarum* strains, suitable for table olive fermentation, are already on the market (11, 31). More recently, in order to offer beneficial health effect to consumers, several cultures of Lactobacillus have been isolated from brines of naturally fermented olives and characterized for their potential probiotic traits (2, 4, 6, 12, 23). Thus, table olives could represent a change to extend the consumption of probiotic foods to certain groups of the population, such as lactose intolerant and persons that need low cholesterol diet. The aims of the present study were to investigate the effect of starters and probiotic strains on (i) the microbiota of green table olives, (ii) the volatile compound formation and (iii) sensory profile of the final products. For these purposes, green table olives from Grossa di Spagna (also known as Bella di Cerignola cv) and Giarraffa cv were artificially inoculated with starters and probiotic strains and analyzed for chemical, microbiological and sensory parameters.

MATERIAL AND METHODS

Olive samples and fermentation procedures

Olives of Giarraffa (G) and Grossa di Spagna (S) cv were kindly provided by a local company, situated in Paternò, Catania, Sicily. After harvesting (September–October), olives were subjected to quality control to remove damaged fruits, washed with tap water and directly immersed in sterilized brine, containing 6% (wv−1) of NaCl. Fermentation was carried out into 20 l total capacity screw-capped PVC vessels, containing proximally 10 kg of olives and 8 l of fresh brine solution. Brine samples were inoculated with starter cultures after 3 days from brining and with potential probiotic strains after 60 days of brining. Overall, five different samples, for each cultivar, Giarraffa (G) and Grossa di Spagna (S), were obtained namely: (G1 and S1) un-inoculated spontaneous sample (control); (G2 and S2) inoculated with probiotic *L. rhamnosus* H25; (G3 and S3) inoculated with commercial probiotic *L. rhamnosus* GG; (G4 and S4) inoculated with *L. plantarum* GC3 plus *L. paracasei* BS21; and (G5 and S5) inoculated with *L. plantarum* GC3 plus *L. paracasei* BS21 plus *L. rhamnosus* H25. All treatments were performed in duplicate (i.e., two fermentation vessels per treatment). Fermentation took place at room temperature (ca. 20–22 °C) for a period of 120 days. During the fermentation, the brine salt concentration was maintained constant at the initial level of 6.0% by periodical additions of coarse salt.

Bacterial strains and culture conditions

Two selected strains, *L. plantarum* GC3 and *L. paracasei* BS21, belonging to DiGeSA microbial collection, previously isolated from naturally fermented table olive brines, characterized for

phenotypic and genotypic traits, and selected for their ability to grow in the presence of oleuropein, hydroxytyrosol, tyrosol and verbascoside (unpublished data), were used as starter cultures. Moreover, two probiotic *L. rhamnosus* strains, the H25 strain (belong to the DiGeSA collection) and the commercial strain LGG (ATCC 53103), were added after 60 days of brining to minimize the competition with starters for nutrients, as previously reported (12). The *L. rhamnosus* H25 strain was previously isolated from cheese and tested for its probiotic traits in previous studies (35, 34, 33). In detail, single frozen concentrated cultures of the starter strains were grown overnight at 32 °C in Man–Rogosa–Sharpe medium broth (MRS, Oxoid, Italy) and re-cultured in the same medium supplemented with 4.5% (w v−1) of NaCl, in order to allow adaptation of strains to the saline environment of the brines (13). Probiotic strains were grown at 37 °C in MRS broth and when the OD600 reached the value of 1.0, cells were harvested (8000 rpm for 10 min), using the microcentrifuge IEC microCL 17 (Thermo Scientific), washed and re-suspended in physiological saline solution (0.9% w v−1 of NaCl). An appropriate volume of starter inoculum (ml), from each individual culture (ratio 1:1), was added, after 7 days of brining, to obtain a final concentration of 8 log colony forming unit (CFU) ml−1. At the same way, appropriate volumes of the probiotic strain suspensions were added in the brines at a final concentration of 7 log CFUml−1. Samples not inoculated were used as control.

Physico-chemical analyses

During the whole fermentation, physico-chemical characteristics of olive brine samples were monitored. Brine samples were routinely analysed for salt concentration 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 (16). Moreover the pH values of brines were detected by pHmeter (H19017,Microprocessor, Hanna Instruments) and lactic acid concentration was determined by enzymatic assay (Megazyme, Poncarale, Italy) at the beginning (7 days), the middle (60 days) and the end of fermentation (120 days) and expressed as g l−1.

Microbiological analyses

Microbial counts

Brine samples (about 10 ml) were analyzed at 0, 7, 14, 21, 30, 60, 63, 75, 90, and 120 days of brining. At each sampling time, brines were serially diluted using sterile quarter-strength Ringer's solution (QRS) and plated in duplicate on the following agar media and conditions: Plate Count Agar (PCA, Sigma,Milan, Italy), incubated at 30 °C for 72 h, to determine the viable mesophilic counts; MRS, anaerobically incubated at 37 °C for 48 h, for LAB counts; Sabouraud Dextrose Agar (SDA, Oxoid) supplemented with chloramphenicol (0.05 g l−1) and incubated at 25 °C for 4 days for yeast counts; Mannitol Salt Agar (MSA,Oxoid), incubated at 32 °C for 48 h, for staphylococci enumeration; and Violet Red Bile Glucose Agar (VRBGA, Difco, Italy), aerobically incubated at 37 °C for 24 h, for total Enterobacteriaceae counts. Results were expressed as log10 CFU per ml (log CFU ml−1).

Isolation and identification of LAB

From each MRS plate of both Giarraffa and Grossa di Spagna brine samples at 120 days of fermentation, 40 colonies were randomly selected, purified, and checked for catalase activity and Gram reaction, and microscopically examined prior to storing in liquid culture using 20%

glycerol at−80 °C. A total of 400 colonies were selected. Total genomic DNA was extracted from LAB overnight cultures following the method described by De los Reyes-Gavilan, Limsowtin, Tailliez, Séchaud, and Acholas (1992) (14). DNA concentration and DNA quality were assessed by measuring optical density at 260 and 280 nm using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). LAB isolates were identified by *rec*A gene multiplex PCR (50). PCR reactions were carried out in a final volume of 20 µl containing 2 µl of DNA, 1× PCR buffer, 1.5 mM MgCl2, 100 µM dNTPs, 1 µM of primers PentF (5-CAG TGG CGC GGT TGA TAT-3), ParaF (5-GTC ACA GGC ATT ACG AAA AC-3) and pREV (5-TCG GGA TTA CCA AAC ATC AC-3), 0.12 µMofprimer PlanF (5-CCG TTT ATG CGG AAC ACC TA-3), and 1 U of Taq polymerase (Invitrogen, Italy). The amplification program was as follows: 30 cycles at 94 °C for 0.5 min, 56 °C for 10 s and 72 °C for 0.5min. An initial denaturation at 94 °C for 3 min and a final extension at 72 °C for 5 min were included. PCR reactions were carried out in a GeneAmp PCR System 2400 (Applied Biosystems, Norwalk, CT, USA). The PCR products were resolved by electrophoresis using 1% agarose gels in TBE buffer (89mMTris–borate, 89mMboric acid, 2mMEDTA; pH 8.0) and visualized after staining with Gel Red Nucleic Acid Stain (Biotium, Italy). LAB isolates were also identified by a previously described PCR assay (53). The PCR reactions were performed in a total volume of 25 µl containing 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 µM each of the four deoxynucleoside triphosphates (dNTP), 2.5 U of Taq polymerase, 10 pmol of each primer PAR (5’-GACGGTAAGATTGGTGA-3’), CAS (5’-ACTGAAGGCGACAAGGA-3’), and RHA (5’-GCGTCAGGTTGGTGTTG-3’), 50 pmol of primer CPR (5’-CAANTGGATNGAACCTGGCTTT-3’), and 25 ng of template DNA. Amplification reactions were performed with the following temperature profiles: 1 cycle at 5 °C for 5 min; 30 cycles at 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 1.5 min; 1 cycle at 72 °C for 7 min. PCR amplicons were analyzed as described above.

Sequence analysis

To confirm the species attribution of the investigated LAB, the 16S rRNA gene was sequenced and compared with those present in the public data libraries GenBank and EZ-Taxon. The BLAST search program was used to determine the closest known relatives http://www.ncbi.nlm.nih.gov/BLAST/; http://ezgenome.ezbiocloud.net/ezg_BLAST).

Sequences with a percentage identity of 98%, or higher, were considered to belong to the same species.

Volatile compound analyses by gas chromatography–mass-spectrometry

Volatile compounds, detected in brine samples at 60, 90 and 120 days of fermentation, were sampled using a SPME extraction. The SUPELCO SPME (Bellefonte, PA) fiber holder and fiber used were coated with divinylbenzene/polydimethylsiloxane (DV/PDMS), 65 µm. Before the first extraction, the fiber was conditioned in the GC injector port at 300 °C for 1 h, according to the manufacturer's recommendation. Ten ml of brine sample were added to a 35 ml vial. Extraction temperature of head-space and time were 40 °C and 20 min, respectively. One gram of NaCl was added to increase extraction rate of volatile compounds. The samples were gently vortexed during extraction using a magnetic stirrer. Fiber exposition was prolonged for 20 min at 40 °C. Thermal desorption was performed in the injector at 230 °C for 1 min (24, 42). The identification of the extracted volatile compounds was carried out using GC–MS instrument (HP GC6890, Hewlett Packard, Palo Alto, CA), and a MS detector (HP MS5973) (29). The gas chromatograph was equipped with a 30 m× 0.25 mm i.d. × 0.25 µm film thickness fused-silica capillary column (DB-WAX J&W Scientific) and the injector temperature was 230 °C. The

conditions were as follows: carrier gas, helium; column flow rate, 1.0 ml min−1; oven temperature program: 2 min at 40 °C, increased at 4 °C min−1 to 50 °C, held 5 min to 50 °C and increased at 4 °C min−1 to 230 °C, held for 10 min at 230 °C. The injector was operated in the split mode with the purge activation time adjusted to 2min. The transfer line was held at 280 °C. The ion source, an electron-impact ionization (EI) type, setup at 70 eV, was held at 230 °C, quadruple at 150 °C and calibration was done by auto-tuning. A ChemStation data system (G1701CA, Hewlett Packard, Palo Alto, CA) was used for data processing. Aroma compounds were identified on the basis of measuring mass spectra by GC–MS and the peak identification was accomplished by comparison of the spectra of reference compounds (NIST/EPA/MSDC Mass Spectral Database, T.G. House, Cambridge, UK), and on the basis of the retention time, compared to the database values for standard reference materials. Confirmation was carried out using a laboratory built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and conditions. All analyses were performed in triplicate and the results were expressed in percent area of total area.

Sensory analysis

To evaluate the sensory differences among the olive samples differently treated, the sensory profile method (52), described by Aponte et al. (2012) (3),was applied on the brine olives of both cultivars at the end of fermentation. A panel of 11 judges (6 females and 5 males, aged between 24 and 30 years) was trained in preliminary sessions, using different samples of canned olives, in order to develop a common vocabulary for the description of the sensory attributes of green table olives and to familiarize themselves with scales and procedures. Each attribute term was extensively described and explained to avoid any doubt about the relevant

meaning. On the basis of the frequency of citation (N60%), fifteen descriptors were selected to be inserted in the card: green color, bright (aspect), green olive aroma, off-odor (odor), crisp, easy stone, juicy (rheological), sweet, acid, bitter, salt (taste), astringent (tactile in mouth), green olive flavor, off-flavor (flavor) and overall. Olive samples were evaluated just once. The different descriptors were quantified using a nine point intensity scale where the number 1 indicates the descriptor absence while the number 9 indicates the full intensity. Evaluations were led in single boxes at the University of Catania sensory analysis laboratory. The order of presentation was randomized among judges and sessions. All data were acquired by a direct computerized registration system (FIZZ Byosistemes. ver. 2.00 M, Couternon, France).

Statistical analysis

All experiments were performed in duplicate and the experimental data were reported as average values and provided with standard deviation. Statistical ANOVA (p N 0.01) and Duncan tests were performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA). Statistical ANOVA was carried out to evaluate significant differences in bacterial growth on different media during the whole fermentation process and on volatile compounds. Moreover, general linear model repeated measures procedures, which provide analysis of variance when the same measurement is made several times on each subject or case, were applied. Specifically, the analysis was performed in order to evaluate differences among fermentation behaviour of the different olive samples belonging to Giarraffa and Grossa di Spagna cv. In order to correlate the brine samples of Giarraffa and Grossa di Spagna cultivars to volatile compounds identified, data obtained at 60, 90 and 120 days of fermentation were subjected to principal component

analysis (PCA), achieving high data compression efficiency of the original data. The sensory data for each attribute were submitted to Analysis of the Variance by software package STATGRAPHICS Centurion XVI using samples as effects. To differentiate the samples the mean values were submitted to the multiple comparison test using the Least Significant Difference (LSD) procedure.

RESULTS

Physico-chemical and microbiological changes during fermentation

The values of pH and microbial counts, expressed as log10 CFU ml−1 of the brine samples, for both Giarraffa and Grossa di Spagna cv are presented in Tables 1 and 2, respectively. Lactic acid trend during fermentation is shown in Figure 3.1. The initial pH values of 5.4 decreased rapidly within the first 14 days in all samples, reaching final values between 3.83 and 4.48. A significant acidification of the brine was observed in spontaneous fermentation samples (G1 and S1), where the pH drop was faster (Tables 3.1 and 3.2). Lactic acid concentration showed an increasing trend in all samples, except for the G3 sample, reaching final values in the range from 2.36 to 5.06 g l−1. The highest final concentration was achieved in spontaneous fermentation G1 sample (Table 3.1). Evaluating microbial count trend in the Giarraffa cv (Table 3.1), viable mesophilic bacteria exhibited a significant increase from the initial level of 3.4 log CFU ml−1 to ca 7.0 log CFU ml−1 in all fermentation samples, with the lowest level in the G1 sample. From the initial level of about 3 log CFU ml−1 lactobacilli significantly increased up to 7 log CFU ml−1 after 21 days of fermentation in G1, G2 and G3 samples, and after 7 days, in samples inoculated with starters (G4 and G5). Yeast population, from the initial value of 2.8

log CFU ml−1, reached, at the 7th day of fermentation, in all samples, a mean value of 6.7 log CFU ml−1. Staphylococci counts exhibited, in all samples, a significant initial increase from 3.7 to ca 7.6 log CFU ml−1, after 7 days of fermentation, a significant decrease at the 60th day of fermentation (mean value of 2.6 log CFU ml−1) and increased to about 5 log CFU ml−1 at the 120th day. Enterobacteriaceae count decreased significantly throughout the fermentation process (Table 3.1). In the Grossa di Spagna cv samples (Table 3.2) the mesophilic counts from the initial level of 4.1 log CFU ml−1 increased during the process reaching a final mean value of 6.8 log CFU ml−1. Lactobacilli counts showed different trends among samples during the first days of fermentations. In details, while in samples S1, S2 and S3 the threshold of 7 log CFU ml−1 is reached at the 30th day of fermentation, in the starter inoculated samples the same value is reached at the 14th day (Table 3.2). As in Giarraffa cv samples, the yeast counts significantly increased at the beginning of the fermentation in all samples of Grossa di Spagna cv, keeping a mean value of 6.8 log CFU ml−1. Staphylococci and Enterobacteriaceae exhibited a similar trend observed in Giarraffa cv samples except for the S1 samples where they showed a significant increase at the end of fermentation (Table 3.2). Evaluating fermentation trends among samples of each cultivar, ANOVA data revealed significant differences between spontaneous and inoculated with starter samples and a similar trend within samples inoculated with probiotic strains, as illustrated in Fig. A, which shows the lactobacilli trend in Grossa di Spagna cv.

Table 3.1 pH value and cell numbers of the principal microbial groups found in brine samples (G1 to G5) of table olives of Giarraffa (G) cultivar.

Brines	Sampling time (days)	pH	Microbial counts expressed as $\log_{10}$ CFU ml^{-1} and standard deviation				
			PCA	MRS	SDA	MSA	VRBGA
G	0	5.45	3.42 ± 0.210	3.06 ± 0.08	2.86 ± 0.13	3.67 ± 0.04	2.50 ± 0.03
G1	7	4.85	$6.75^{bc} \pm 0.07$	$4.52^{a} \pm 0.22$	$6.57^{cd} \pm 0.30$	$7.62^{f} \pm 0.06$	$5.30^{e} \pm 0.01$
	14	4.15	$7.20^{cd} \pm 0.04$	$6.49^{b} \pm 0.02$	$6.37^{bc} \pm 0.09$	$5.57^{e} \pm 0.04$	$2.61^{d} \pm 0.05$
	21	3.70	$7.67^{d} \pm 0.05$	$7.38^{d} \pm 0.11$	$6.91^{e} \pm 0.01$	$1.94^{c} \pm 0.08$	$1.74^{bc} \pm 0.10$
	30	3.91	$7.47^{d} \pm 0.05$	$7.18^{d} \pm 0.04$	$7.49^{f} \pm 0.08$	$1.87^{c} \pm 0.14$	$1.99^{c} \pm 0.03$
	60	3.83	$6.00^{a} \pm 0.01$	$6.50^{b} \pm 0.03$	$6.06^{ab} \pm 0.17$	$1.38^{a} \pm 0.08$	$1.38^{a} \pm 0.23$
	63	3.66	$6.58^{abc} \pm 0.08$	$6.90^{c} \pm 0.04$	$6.13^{ab} \pm 0.07$	$2.02^{c} \pm 0.04$	$1.72^{b} \pm 0.08$
	75	3.56	$6.69^{bc} \pm 0.12$	$6.55^{b} \pm 0.07$	$7.38^{f} \pm 0.11$	$1.61^{b} \pm 0.08$	$1.33^{a} \pm 0.13$
	90	3.73	$6.77^{bc} \pm 0.78$	$7.31^{d} \pm 0.15$	$6.69^{de} \pm 0.09$	$1.35^{a} \pm 0.11$	$1.87^{bc} \pm 0.16$
	120	3.83	$6.25^{ab} \pm 0.07$	$7.41^{d} \pm 0.15$	$6.00^{a} \pm 0.03$	$5.00^{d} \pm 0.80$	$1.67^{b} \pm 0.08$
G2	7	5.30	$7.06^{b} \pm 0.08$	$5.06^{a} \pm 0.09$	$6.52^{b} \pm 0.03$	$7.42^{f} \pm 0.17$	$5.64^{c} \pm 0.05$
	14	4.64	$7.07^{b} \pm 0.09$	$6.27^{b} \pm 0.05$	$6.53^{b} \pm 0.04$	$5.64^{e} \pm 0.05$	$2.62^{b} \pm 0.12$
	21	4.43	$7.88^{d} \pm 0.01$	$7.55^{f} \pm 0.14$	$6.80^{c} \pm 0.13$	$1.68^{a} \pm 0.00$	$1.83^{a} \pm 0.22$
	30	4.54	$7.37^{c} \pm 0.19$	$7.26^{de} \pm 0.66$	$7.51^{d} \pm 0.08$	$1.57^{a} \pm 0.04$	$1.72^{a} \pm 0.21$
	60	4.57	$5.99^{a} \pm 0.01$	$6.46^{bc} \pm 0.09$	$6.56^{bc} \pm 0.13$	$3.29^{b} \pm 0.16$	$1.79^{a} \pm 0.16$
	63	4.50	$7.57^{c} \pm 0.04$	$6.43^{bc} \pm 0.22$	$5.35^{a} \pm 0.14$	$3.99^{c} \pm 0.13$	$1.50^{a} \pm 0.08$
	75	4.41	$7.07^{b} \pm 0.16$	$7.21^{d} \pm 0.19$	$7.27^{d} \pm 0.02$	$4.21^{cd} \pm 0.08$	$1.65^{a} \pm 0.43$
	90	4.37	$7.45^{c} \pm 0.01$	$7.52^{ef} \pm 0.08$	$7.50^{d} \pm 0.10$	$4.35^{d} \pm 0.00$	$2.44^{b} \pm 0.06$
	120	4.44	$7.30^{bc} \pm 0.15$	$6.65^{c} \pm 0.07$	$7.29^{d} \pm 0.16$	$4.36^{d} \pm 0.26$	$1.83^{a} \pm 0.23$
G3	7	5.31	$7.35^{de} \pm 0.07$	$5.16^{a} \pm 0.09$	$6.99^{e} \pm 0.01$	$7.13^{h} \pm 0.04$	$5.39^{c} \pm 0.06$
	14	5.00	$7.39^{de} \pm 0.07$	$6.30^{c} \pm 0.09$	$6.33^{a} \pm 0.01$	$5.47^{g} \pm 0.05$	$2.44^{b} \pm 0.07$
	21	4.67	$7.74^{f} \pm 0.13$	$7.29^{f} \pm 0.14$	$6.63^{bc} \pm 0.14$	$1.23^{a} \pm 0.01$	$1.72^{a} \pm 0.08$
	30	4.71	$7.46^{e} \pm 0.13$	$7.06^{e} \pm 0.07$	$7.49^{g} \pm 0.06$	$1.55^{b} \pm 0.30$	$1.51^{a} \pm 0.23$
	60	4.51	$5.92^{a} \pm 0.05$	$6.62^{d} \pm 0.12$	$6.42^{ab} \pm 0.12$	$1.87^{c} \pm 0.16$	$1.50^{a} \pm 0.08$
	63	4.36	$6.78^{b} \pm 0.07$	$7.00^{e} \pm 0.01$	$6.67^{c} \pm 0.07$	$2.68^{d} \pm 0.11$	$1.71^{a} \pm 0.07$
	75	4.30	$6.93^{bc} \pm 0.04$	$6.97^{e} \pm 0.04$	$6.74^{cd} \pm 0.06$	$2.87^{d} \pm 0.04$	$1.78^{a} \pm 0.15$
	90	4.42	$7.38^{de} \pm 0.14$	$6.33^{c} \pm 0.14$	$6.93^{de} \pm 0.04$	$3.39^{e} \pm 0.00$	$1.55^{a} \pm 0.30$
	120	4.47	$7.15^{cd} \pm 0.21$	$5.98^{b} \pm 0.03$	$7.21^{f} \pm 0.13$	$4.39^{f} \pm 0.12$	$1.70^{a} \pm 0.21$
G4	7	5.50	$7.75^{e} \pm 0.07$	$7.49^{f} \pm 0.02$	$6.50^{ab} \pm 0.01$	$7.47^{f} \pm 0.05$	$5.31^{e} \pm 0.01$
	14	5.07	$7.95^{e} \pm 0.07$	$7.83^{g} \pm 0.08$	$6.23^{a} \pm 0.16$	$7.38^{f} \pm 0.03$	$2.57^{b} \pm 0.02$
	21	4.72	$7.87^{e} \pm 0.04$	$7.83^{g} \pm 0.08$	$7.20^{c} \pm 0.04$	$5.05^{d} \pm 0.07$	$2.85^{bc} \pm 0.07$
	30	4.76	$6.95^{bc} \pm 0.08$	$7.06^{d} \pm 0.08$	$7.14^{c} \pm 0.09$	$2.06^{a} \pm 0.08$	$2.58^{b} \pm 0.03$
	60	4.82	$6.09^{a} \pm 0.12$	$7.29^{e} \pm 0.16$	$7.40^{c} \pm 0.03$	$3.87^{c} \pm 0.03$	$4.48^{d} \pm 0.11$
	63	4.65	$7.07^{cd} \pm 0.11$	$6.66^{c} \pm 0.07$	$7.26^{c} \pm 0.21$	$3.39^{b} \pm 0.20$	$1.60^{a} \pm 0.23$
	75	4.65	$6.87^{bc} \pm 0.04$	$6.01^{b} \pm 0.04$	$6.54^{b} \pm 0.10$	$5.01^{d} \pm 0.26$	$1.60^{a} \pm 0.21$
	90	4.44	$7.31^{d} \pm 0.23$	$5.73^{a} \pm 0.05$	$7.29^{c} \pm 0.16$	$5.22^{d} \pm 0.18$	$2.87^{bc} \pm 0.03$
	120	4.42	$6.74^{b} \pm 0.04$	$6.95^{d} \pm 0.0$	$6.53^{b} \pm + 0.18$	$5.67^{e} \pm 0.10$	$1.49^{a} \pm 0.08$
G5	7	4.85	$7.73^{d} \pm 0.20$	$7.71^{e} \pm 0.12$	$6.6^{d} \pm 0.03$	$8.28^{h} \pm 0.03$	$5.06^{e} \pm 0.08$
	14	4.58	$7.40^{e} \pm 0.14$	$7.11^{d} \pm 0.15$	$5.87^{c} \pm 0.04$	$5.74^{g} \pm 0.08$	$2.79^{c} \pm 0.03$
	21	4.47	$7.84^{e} \pm 0.09$	$7.94^{f} \pm 0.08$	$7.13^{ef} \pm 0.08$	$3.98^{e} \pm 0.03$	$3.51^{d} \pm 0.04$
	30	4.65	$6.95^{ab} \pm 0.07$	$6.36^{b} \pm 0.05$	$6.98^{e} \pm 0.03$	$2.75^{c} \pm 0.07$	$2.79^{c} \pm 0.02$
	60	4.70	$6.85^{a} \pm 0.04$	$6.01^{a} \pm 0.04$	$7.21^{f} \pm 0.03$	$2.65^{c} \pm 0.07$	$2.74^{c} \pm 0.06$
	63	4.75	$7.52^{cd} \pm 0.10$	$6.73^{c} \pm 0.06$	$4.66^{a} \pm 0.07$	$2.34^{b} \pm 0.21$	$1.83^{b} \pm 0.21$
	75	4.73	$7.12^{abc} \pm 0.05$	$6.53^{b} \pm 0.07$	$4.88^{b} \pm 0.04$	$3.02^{d} \pm 0.09$	$1.77^{b} \pm 0.14$
	90	4.56	7.29 ± 0.16	$6.93^{d} \pm 0.03$	$7.23^{f} \pm 0.06$	$4.60^{ef} \pm 0.08$	$1.34^{a} \pm 0.27$
	120	4.48	$7.23^{abc} \pm 0.07$	$7.61^{e} \pm 0.08$	$7.17^{ef} \pm 0.12$	$5.25^{f} \pm 0.00$	$1.87^{b} \pm 0.15$

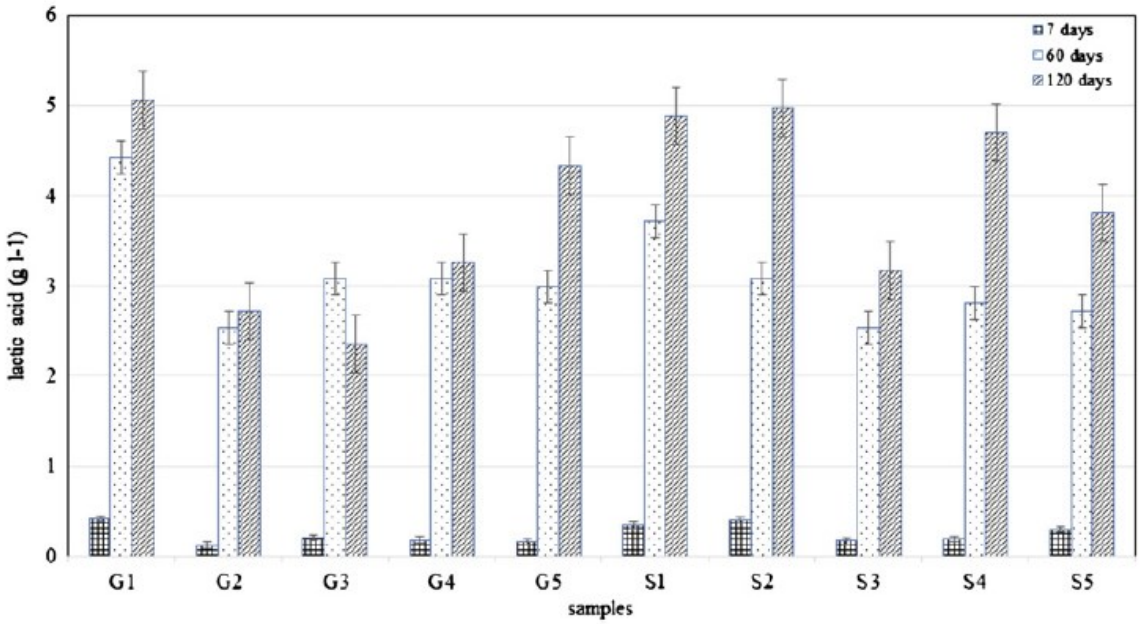
Mean values of two independent samples taken throughout brine fermentation; lowercase (a, b, etc) letters in the same column indicate different statistical significances according to Duncan test at p value of $p < 0.01$.

Table 3.2 pH value and cell numbers of the principal microbial groups found in brine samples (S1 to S5) of table olives of Grossa di Spagna (S) cultivar.

Brines	Sampling time (days)	pH	Microbial counts expressed as $\log_{10}$ CFU ml^{-1} and standard deviation				
			PCA	MRS	SDA	MSA	VRBGA
S	0	5.44	4.19 ± 0.06	3.40 ± 0.07	2.77 ± 0.05	4.26 ± 0.08	3.36 ± 0.03
S1	7	5.00	$6.70^{ab} \pm 0.14$	$4.92^{a} \pm 0.12$	$6.84^{bc} \pm 0.06$	$7.39^{f} \pm 0.06$	$5.12^{d} \pm 0.06$
	14	4.45	$7.87^{c} \pm 0.04$	$6.48^{b} \pm 0.03$	$6.37^{a} \pm 0.09$	$5.47^{e} \pm 0.05$	$2.39^{b} \pm 0.07$
	21	3.86	$7.44^{bc} \pm 0.06$	$6.79^{c} \pm 0.14$	$6.61^{ab} \pm 0.05$	$1.34^{a} \pm 0.01$	$1.66^{a} \pm 0.14$
	30	4.00	$6.9^{ab} \pm 0.02$	$7.08^{de} \pm 0.11$	$7.37^{de} \pm 0.04$	$1.45^{ab} \pm 0.16$	$1.34^{a} \pm 0.16$
	60	3.85	$6.68^{ab} \pm 0.11$	$7.07^{de} \pm 0.16$	$7.67^{e} \pm 0.07$	$1.72^{b} \pm 0.06$	$1.40^{a} \pm 0.07$
	63	3.69	$6.97^{ab} \pm 0.05$	$7.12^{e} \pm 0.16$	$7.37^{de} \pm 0.10$	$1.66^{b} \pm 0.15$	$1.72^{a} \pm 0.31$
	75	3.59	$6.99^{ab} \pm 0.97$	$7.07^{de} \pm 0.11$	$7.42^{de} \pm 0.16$	$2.13^{c} \pm 0.18$	$1.34^{a} \pm 0.19$
	90	3.84	$6.95^{ab} \pm 0.07$	$6.91^{cde} \pm 0.06$	$7.21^{cd} \pm 0.19$	$2.07^{c} \pm 0.11$	$4.21^{c} \pm 0.19$
	120	3.98	$6.50^{a} \pm 0.03$	$6.83^{cd} \pm 0.02$	$6.84^{bc} \pm 0.04$	$2.98^{d} \pm 0.03$	$4.49^{c} \pm 0.36$
S2	7	5.23	$7.02^{c} \pm 0.03$	$5.05^{a} \pm 0.06$	$7.07^{d} \pm 0.09$	$7.45^{e} \pm 0.07$	$5.15^{d} \pm 0.07$
	14	4.85	$7.95^{e} \pm 0.06$	$6.59^{c} \pm 0.12$	$6.21^{a} \pm 0.16$	$5.73^{d} \pm 0.08$	$2.55^{c} \pm 0.46$
	21	4.50	$7.29^{d} \pm 0.11$	$6.93^{d} \pm 0.08$	$6.70^{abc} \pm 0.04$	$2.44^{bc} \pm 0.14$	$1.35^{a} \pm 0.15$
	30	4.62	$6.95^{c} \pm 0.08$	$7.23^{e} \pm 0.03$	$7.45^{d} \pm 0.07$	$1.55^{a} \pm 0.15$	$1.50^{ab} \pm 0.08$
	60	4.47	$6.13^{a} \pm 0.18$	$5.92^{b} \pm 0.04$	$6.34^{ab} \pm 0.08$	$1.49^{a} \pm 0.23$	$1.49^{ab} \pm 0.23$
	63	4.28	$6.50^{b} \pm 0.05$	$7.18^{de} \pm 0.26$	$6.79^{bc} \pm 0.05$	$1.72^{ab} \pm 0.37$	$1.66^{ab} \pm 0.13$
	75	4.25	$7.53^{d} \pm 0.10$	$7.27^{e} \pm 0.18$	$7.47^{d} \pm 0.12$	$1.61^{ab} \pm 0.08$	$1.51^{ab} \pm 0.24$
	90	4.48	$7.45^{d} \pm 0.04$	$7.23^{e} \pm 0.04$	$7.49^{d} \pm 0.10$	$1.88^{ab} \pm 0.14$	$1.93^{b} \pm 0.07$
	120	4.35	$7.32^{d} \pm 0.05$	$6.89^{d} \pm 0.04$	$6.38^{ab} \pm 0.11$	$2.70^{c} \pm 0.07$	$1.88^{b} \pm 0.07$
S3	7	5.13	$6.80^{a} \pm 0.13$	$4.59^{a} \pm 0.13$	$6.89^{ab} \pm 0.13$	$7.05^{f} \pm 0.06$	$4.95^{e} \pm 0.07$
	14	4.65	$7.62^{b} \pm 0.09$	$6.51^{c} \pm 0.19$	$6.36^{a} \pm 0.06$	$5.29^{e} \pm 0.08$	$2.83^{d} \pm 0.22$
	21	4.47	$7.50^{b} \pm 0.04$	$7.00^{d} \pm 0.12$	$6.56^{a} \pm 0.05$	$1.76^{ab} \pm 0.08$	$1.61^{abc} \pm 0.25$
	30	4.56	$7.05^{ab} \pm 0.07$	$7.21^{de} \pm 0.08$	$7.49^{b} \pm 0.10$	$1.50^{a} \pm 0.10$	$1.71^{abc} \pm 0.08$
	60	4.63	$6.46^{a} \pm 0.09$	$6.01^{b} \pm 0.09$	$6.96^{ab} \pm 0.04$	$3.39^{c} \pm 0.12$	$1.77^{bc} \pm 0.14$
	63	4.35	$7.05^{ab} \pm 0.08$	$7.10^{d} \pm 0.28$	$6.28^{a} \pm 0.24$	$2.81^{bc} \pm 0.05$	$1.40^{a} \pm 0.20$
	75	4.28	$7.15^{ab} \pm 0.21$	$7.51^{e} \pm 0.16$	$7.41^{b} \pm 0.12$	$2.98^{bc} \pm 0.03$	$1.93^{c} \pm 0.08$
	90	4.34	$7.50^{b} \pm 0.25$	$7.07^{d} \pm 0.02$	$6.49^{a} \pm 0.04$	$4.04^{cde} \pm 0.04$	$1.78^{bc} \pm 0.18$
	120	4.45	$6.94^{ab} \pm 0.03$	$6.14^{b} \pm 0.20$	$6.68^{a} \pm 0.10$	$4.87^{de} \pm 0.05$	$1.49^{ab} \pm 0.07$
S4	7	4.88	$6.69^{ab} \pm 0.10$	$6.89^{bc} \pm 0.15$	$6.84^{bc} \pm 0.05$	$7.38^{f} \pm 0.02$	$5.11^{d} \pm 0.02$
	14	4.25	$7.87^{c} \pm 0.11$	$7.10^{d} \pm 0.03$	$6.36^{a} \pm 0.06$	$5.46^{e} \pm 0.05$	$2.39^{b} \pm 0.29$
	21	4.18	$7.44^{bc} \pm 0.20$	$6.88^{bc} \pm 0.02$	$6.61^{ab} \pm 0.07$	$1.34^{a} \pm 0.14$	$1.66^{a} \pm 0.30$
	30	4.41	$6.99^{ab} \pm 0.06$	$7.55^{e} \pm 0.17$	$7.37^{de} \pm 0.03$	$1.45^{ab} \pm 0.15$	$1.73^{a} \pm 0.08$
	60	4.48	$6.68^{ab} \pm 0.12$	$7.36^{e} \pm 0.26$	$7.66^{e} \pm 0.20$	$1.71^{b} \pm 0.12$	$1.40^{a} \pm 0.30$
	63	4.15	$6.96^{ab} \pm 0.12$	$6.73^{b} \pm 0.06$	$7.37^{de} \pm 0.04$	$1.65^{b} \pm 0.21$	$1.72^{a} \pm 0.15$
	75	4.16	$6.99^{ab} \pm 0.07$	$6.76^{b} \pm 0.09$	$7.41^{de} \pm 0.17$	$2.12^{c} \pm 0.16$	$1.34^{a} \pm 0.11$
	90	4.32	$6.95^{ab} \pm 0.01$	$6.61^{a} \pm 0.08$	$7.37^{de} \pm 0.23$	$2.07^{c} \pm 0.12$	$1.21^{a} \pm 0.22$
	120	4.26	$6.50^{a} \pm 0.02$	$6.59^{a} \pm 0.02$	$6.84^{bc} \pm 0.01$	$2.97^{d} \pm 0.06$	$1.83^{a} \pm 0.25$
S5	7	4.70	$7.21^{bc} \pm 0.01$	$5.60^{a} \pm 0.06$	$5.98^{a} \pm 0.02$	$7.08^{f} \pm 0.06$	$3.67^{e} \pm 0.03$
	14	4.20	$7.45^{cd} \pm 0.07$	$7.38^{d} \pm 0.11$	$6.65^{bc} \pm 0.07$	$5.14^{e} \pm 0.09$	$2.38^{d} \pm 0.21$
	21	4.04	$7.35^{bcd} \pm 0.09$	$6.89^{c} \pm 0.01$	$6.53^{b} \pm 0.07$	$1.44^{a} \pm 0.08$	$1.78^{ab} \pm 0.16$
	30	4.23	$7.79^{e} \pm 0.15$	$6.26^{b} \pm 0.08$	$6.95^{bcd} \pm 0.07$	$1.23^{a} \pm 0.32$	$1.66^{a} \pm 0.16$
	60	4.29	$7.50^{d} \pm 0.14$	$6.46^{b} \pm 0.15$	$7.31^{de} \pm 0.15$	$3.42^{d} \pm 0.06$	$2.15^{bc} \pm 0.21$
	63	4.20	$7.16^{b} \pm 0.17$	$6.93^{c} \pm 0.04$	$7.34^{de} \pm 0.27$	$1.49^{a} \pm 0.23$	$1.94^{ab} \pm 0.06$
	75	4.30	$7.86^{e} \pm 0.05$	$6.89^{c} \pm 0.04$	$7.48^{e} \pm 0.17$	$2.82^{c} \pm 0.06$	$1.76^{a} \pm 0.29$
	90	4.34	$7.47^{cd} \pm 0.21$	$7.10^{c} \pm 0.20$	$7.23^{de} \pm 0.12$	$2.47^{b} \pm 0.10$	$2.74^{d} \pm 0.06$
	120	4.29	$6.79^{a} \pm 0.02$	$6.93^{c} \pm 0.06$	$7.07^{cde} \pm 0.10$	$2.67^{bc} \pm 0.03$	$1.96^{ab} \pm 0.13$

Mean values of two independent samples taken throughout brine fermentation; lowercase (a, b, etc) letters indicate different statistical significances according to Duncan test at p value of $p < 0.01$.

Figure 3.1 Change in the concentration of lactic acid (g l−1) in the brine samples of Giarraffa and Grossa di Spagna cultivars during fermentation.

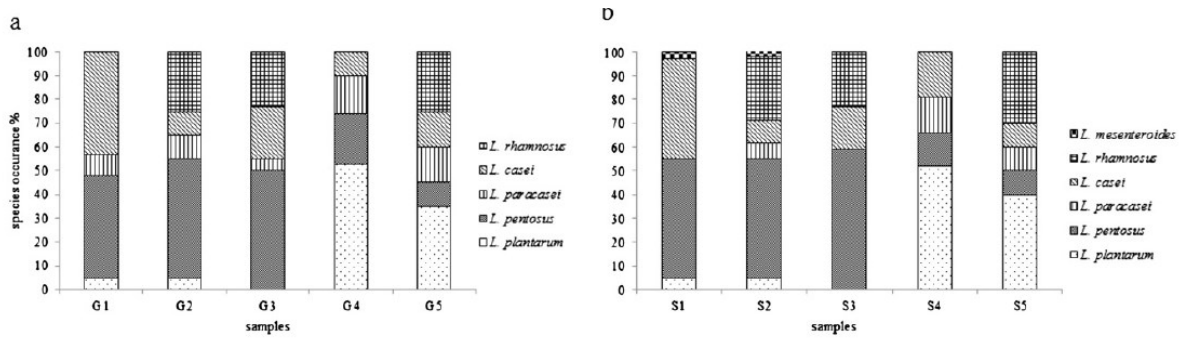


Occurrence of LAB species in brine samples

A total of 400 isolates were recovered after 120 days of fermentation from olives of Giarraffa and Grossa di Spagna cv, and 397 isolates (198 from Giarraffa cv and 199 from Grossa di Spagna cv) were considered LAB for their gram positive, non-spore forming, catalase negative and morphology and were mostly successfully identified by multiplex PCR amplifications as *L. plantarum*, *L. pentosus*, *L. paracasei*, *L. rhamnosus* and *L. casei*. Frequency isolation, expressed as percentage of these species in brine samples of Giarraffa and Grossa di Spagna cv, is reported in Figure 3.2a and b. Overall, results showed that *L. plantarum* was the most frequent species found in brine samples inoculated with starter (G4, G5 and S4, S5),while *L. pentosus* was the most frequent species isolated in spontaneous fermentation brine samples (G1, S1) and in brine samples inoculated with *L. rhamnosus* strains (G2, G3 and S2, S3). It is interesting

to note that *L. plantarum* species was not detected in any samples inoculated with the *L. rhamnosus* GG strain. In brine samples of both olive cultivars *L. rhamnosus* was revealed only in samples in which it was deliberatively added (G2, G3, G5 and S2, S3, S5) while *L. casei* was revealed in all brine samples (Figure 3.2a and b). *L. paracasei* was detected in all samples of Giarraffa cv. while in Grossa di Spagna cv, it did not appear neither in spontaneous sample (S1) nor in LGG inoculated sample (S3). Only 5 strains, occurring in S1 sample, were not identified by multiplex PCR and were ascribed, by sequencing, to *Leuconostoc mesenteroides* species with 99% of similarity.

Figure 3.2. Species occurrence, expressed as percentage, in brine samples of cv Giarraffa (a) and Grossa di Spagna (b) cultivars during fermentation.



Volatile compounds

The analysis of volatile compounds from brines of Giarraffa and Grossa di Spagna cv was performed using SPME-GC–MS, and results are reported in Tables 3.3 and 3.4. The assessment allowed the identification of 37 compounds, as acids, alcohols, aldehydes, esters, ketones and phenols. In detail, in Giarraffa samples, thirty-five different compounds were revealed and twenty-four in the Grossa di Spagna one. Evaluating samples of Giarraffa cv, at 60 days of fermentation the samples G1, G2 and G3 exhibited high value of alcohol compounds (55% of total area), such as ethanol, isoamyl alcohol and phenylethyl alcohols, followed by ester (35% of total area) and phenol (4% of total area) compounds. After 90 days of fermentation a higher decrease of the ethanol was registered in samples inoculated with *L. rhamnosus* strains (G2 and G3), compared to spontaneous sample (G1). The sample inoculated with starters (G4) revealed a decrease in ethanol content, while the sample inoculated with starter and probiotic strains (G5) exhibited, at the 20th day of fermentation, an increase (Table 3.3). Upon evaluating ester compounds, although they were detected at high level in all samples of Giarraffa cv, after 60 days of fermentation, their level significantly decreased through the fermentation. The most abundant compounds were the ethyl-acetate and the ethyl-butanoate. Phenol content significantly increased during the fermentation process in samples G4, G5 and G2. Propionic acid decreased from the 60th day to the 120th day, in all samples inoculated with probiotic cultures (G2, G3 and G5), while acetic acid increased only in spontaneous sample (G1). Aldehydes and ketones showed a high increase only in the G2 sample; in particular, 3-octanal and 3-octanone compounds. Grossa di Spagna samples showed a different trend. In particular, alcoholic compounds displayed a strong reduction after inoculums of probiotic cultures, decreasing from 67.5% to 11.7% in S2 and S3 samples,

respectively. The samples inoculated with starters (S4 and S5) exhibited, at the 60th day of fermentation, low alcohol compounds (2.8% of total area) respect to the amount detected on the un-inoculated sample S2. In these samples ethanol and phenylethyl alcohol increased at the end of the process, reaching higher value in S5 (Table 3.3). Ester compounds were detected at high level in all samples of Grossa di Spagna cv, after 60 days of fermentation, with the highest level both in S4 and S5 samples (64.4%). Their level significantly decreased through the fermentation. Only in S5 sample was found at the end of fermentation 18.3% of ester compounds, especially ethyl succinate and isoamylpropionate. The total phenol content was lower than that found in Giarraffa cv with the highest values in S5 and S3 samples. Volatile acids exhibited the same trends of Giarraffa cv in the spontaneous fermentation with a substantial increase in all samples except in the S5 sample. Figure 3.3 shows the PCA plot of distribution of table olive samples from Giarraffa (G1, G5) and Grossa di Spagna (S1, S5) cv in the PC1–PC2 plane. Based on the loadings (data not shown), PC1, that represent the 27.6% of the variability, can be viewed as an esters and ketones factor, since the higher contribution comes from ethyl-lactate and acetoin compounds. The second principal component (PC2 variance 19.8%) represents the acid and alcohol compounds, since their loadings are mostly associated to 2-butanoic acid and 1-eptanol compounds. Score plots are effective in showing the difference among samples and in separating some of them in the graphs. In detail, the G1 sample has had a positive contribution both on PC1 and on PC2 planes, while the samples S1 and S2 were characterized by a positive contribution of esters and ketones and a negative contribution of acids and alcohols.

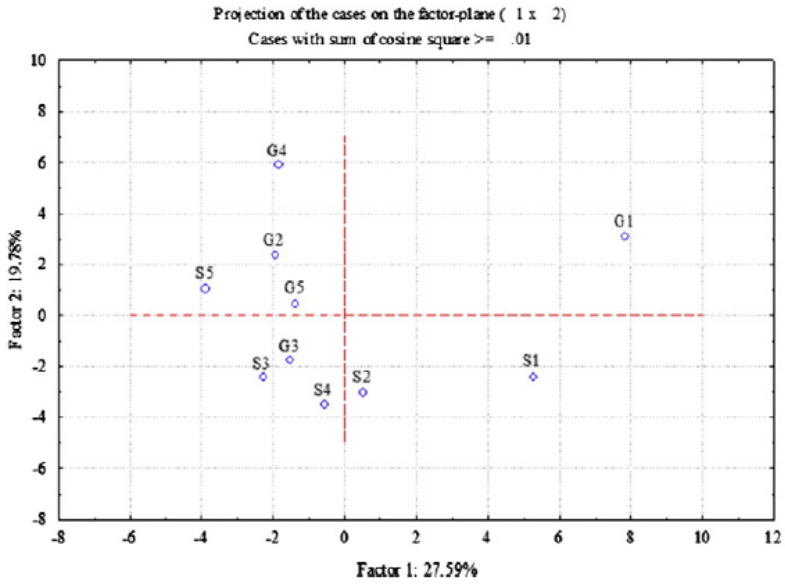
Table 3.3 Volatile compound area (expressed as percent area of total area) of Giarraffa cultivar samples (G1 to G5) detected at 60, 90 and 120 days.

Giarraffa cv		G1			G2			G3			G4			G5		
		Days			Days			Days			Days			Days		
Compounds	RT*	60	90	120	60	90	120	60	90	120	60	90	120	60	90	120
Acids		1.81	4.53	3.84	1.81	33.75	11.56	1.81	41.59	34.46	44.55	25.81	8.04	44.55	34.94	0.66
Acetic acid	25.04	1.10^{c}	2.72^{d}	2.89^{d}	1.10^{c}	2.17^{d}	0.32^{b}	1.10^{c}	0.06^{a}	0.42^{b}	2.46^{d}	2.15^{d}	0.48^{b}	2.46^{d}	2.32^{d}	nd
Propionic acid	30.20	0.61^{a}	1.71^{ab}	0.79^{a}	0.61^{a}	31.21^{d}	10.49^{b}	0.61^{a}	41.03^{e}	33.48^{d}	18.29^{c}	19.56^{c}	5.77^{b}	18.29^{c}	31.49^{d}	0.30^{a}
Isobutyric acid	31.94	0.11^{a}	0.10^{a}	nd	0.11^{a}	0.26^{b}	0.46^{c}	0.11^{a}	0.28^{b}	0.48^{c}	nd	0.12^{a}	0.32^{bc}	nd	0.40^{c}	0.22^{b}
Butanoic acid	35.29	nd	nd	nd	nd	0.11^{a}	0.30^{a}	nd	0.22^{a}	0.08^{a}	23.81^{d}	2.48^{c}	1.47^{c}	23.81^{d}	0.72^{b}	0.14^{a}
Alcohol		55.44	51.31	62.47	55.44	12.50	10.27	55.44	13.08	15.76	11.19	8.36	11.90	11.19	9.24	35.59
Ethanol	3.68	46.04^{d}	27.71^{c}	43.91^{d}	46.04^{d}	2.25^{b}	0.58^{a}	46.04^{d}	2.83^{b}	0.34^{a}	3.26^{b}	1.86^{b}	0.62^{a}	3.26^{b}	nd	20.14^{c}
Isoamylalcohol	12.50	5.30^{d}	11.61^{g}	9.11^{f}	5.30^{d}	3.72^{c}	5.29^{d}	5.30^{d}	6.54^{e}	8.01	4.80^{cd}	2.65^{b}	3.50^{c}	4.80^{cd}	0.52^{a}	4.12^{c}
2-Heptanol	18.14	0.23^{b}	0.29^{b}	0.89^{e}	0.23^{b}	0.38^{c}	0.43	0.23^{b}	0.28^{b}	0.33^{bc}	nd	nd	0.49^{d}	nd	0.11^{a}	0.06^{a}
1-Hexanol	19.98	0.89^{c}	1.29^{d}	1.32^{d}	0.89^{c}	0.14^{a}	0.22^{b}	0.89^{c}	0.33^{b}	0.09^{a}	nd	nd	0.29^{b}	nd	0.09^{a}	0.18^{a}
cis Hexen 1 ol	21.58	0.15^{b}	0.35^{c}	0.52^{d}	0.15^{b}	0.40^{c}	0.09^{a}	0.15^{b}	0.13^{b}	0.06^{a}	nd	nd	0.45^{cd}	nd	0.09^{a}	0.41^{c}
3-Octenol	25.43	nd	1.12^{d}	0.51^{c}	nd	0.02^{a}	0.08^{a}	nd	nd	0.04^{a}	nd	nd	0.55^{c}	nd	0.22^{b}	0.05^{a}
1-Hexanol 2-ethyl	27.78	0.13^{a}	0.38^{c}	0.17^{ab}	0.13^{a}	1.18^{d}	0.20^{ab}	0.13^{a}	0.06^{a}	0.08^{a}	nd	nd	0.24^{b}	nd	0.29^{b}	0.18^{ab}
Phenylethyl alcohol	49.80	2.17^{a}	7.46^{e}	5.21^{c}	2.17^{a}	4.08^{b}	2.75^{a}	2.17^{a}	2.59^{a}	6.53^{d}	2.09^{a}	3.15^{a}	5.15^{c}	2.09^{a}	7.49^{e}	9.80^{f}
Aldeydes		1.98	2.30	1.34	1.98	4.46	5.96	1.98	0.15	2.50	3.01	1.35	1.31	3.01	1.65	2.30
Octanal	15.34	0.24^{b}	0.33^{b}	0.13^{a}	0.24^{b}	0.80^{c}	0.06^{a}	0.24^{b}	nd	0.34^{b}	0.27^{b}	0.27^{b}	0.25^{b}	0.27^{b}	0.22^{b}	0.08^{a}
Nonanal	21.26	0.70^{d}	0.47^{c}	0.37^{b}	0.70^{d}	1.90^{g}	1.01^{f}	0.70^{d}	0.06^{a}	1.15^{f}	1.77	0.15^{a}	nd	1.77^{g}	0.90^{e}	0.61^{d}
3-Octanal	22.24	nd	0.37^{b}	0.14^{a}	nd	0.03^{a}	3.66^{c}	nd	0.01^{a}	0.09^{a}	nd	nd	0.39^{b}	nd	0.05^{a}	0.09^{a}
Decanal	27.40	0.92^{c}	0.66^{b}	0.50^{a}	0.92^{c}	1.68^{d}	0.89^{c}	0.92^{c}	0.90	0.86^{c}	0.82^{c}	0.69^{b}	0.54^{ab}	0.82^{c}	0.38^{a}	0.83^{c}
Benzaldehyde	28.32	0.11	0.48	0.21	0.11	0.06	0.33	0.11	nd	0.06	0.14	0.12	0.12	0.14	0.10	0.70
Esters		35.52	26.20	14.99	35.52	29.02	15.12	35.52	30.53	7.45	25.74	15.36	10.96	25.74	22.99	1.72
Ethyl-acetate	2.97	24.91^{g}	10.87^{f}	5.27^{e}	24.91^{g}	1.15^{b}	1.80^{c}	24.91^{g}	2.70^{d}	0.35^{a}	10.58^{f}	2.23^{c}	0.30^{a}	10.58^{f}	2.02^{c}	nd
Methyl-propanoate	3.15	nd	nd	nd	nd	2.87^{d}	4.66^{f}	nd	3.53^{e}	1.51^{c}	nd	0.39^{a}	1.04^{b}	nd	nd	nd
Ethyl-propanoate	3.89	nd	nd	nd	nd	11.70^{d}	2.26^{b}	nd	15.59^{f}	0.41^{a}	nd	0.47^{a}	0.94^{a}	nd	8.71^{c}	nd
Ethyl-butanoate	4.66	nd	nd	nd	nd	0.84^{a}	nd	nd	nd	nd	12.45^{d}	10.21^{c}	3.03^{b}	12.45^{d}	nd	nd
Isoamyl-propionate	10.34	nd	nd	nd	nd	5.92^{c}	1.20^{a}	nd	0.96^{a}	1.30^{a}	1.10^{a}	1.12^{a}	1.15^{a}	1.10^{a}	2.09^{b}	nd
Ethyl-hexanoate	12.68	4.12^{c}	3.30^{c}	1.85^{b}	4.12^{c}	0.35^{a}	nd	4.12^{c}	nd	nd	nd	nd	nd	nd	5.34^{d}	nd
Ethyl-lactate	19.17	2.04^{b}	3.98^{c}	4.22^{c}	2.04^{b}	0.45^{a}	0.17^{a}	2.04^{b}	0.80^{a}	0.14^{a}	nd	nd	0.21^{a}	nd	0.40^{a}	0.05^{a}
Ethyl-octanoate	23.90	0.09^{a}	0.04^{a}	0.12^{a}	0.09^{a}	0.04^{a}	0.07^{a}	0.09^{a}	3.13^{b}	0.05^{a}	nd	nd	0.05^{a}	nd	nd	nd
Phenyl-ethyl-acetate	44.63	nd	1.61^{b}	nd	nd	0.18^{a}	nd	nd	0.20^{a}	0.07^{a}	0.12^{a}	nd	nd	0.12^{a}	0.17^{a}	0.29^{a}
Benzenepropanoic acid ethyl-ester	48.16	3.00^{c}	2.97^{c}	1.36^{b}	3.00^{c}	3.26^{c}	0.66^{a}	3.00^{c}	2.02^{b}	1.65^{b}	0.76^{a}	0.81^{a}	1.29^{b}	0.76^{a}	1.63^{b}	0.78^{a}
Ketones		0.61	0.64	0.92	0.61	0.03	9.17	0.61	0.00	0.29	0.00		1.12	0.00	0.31	0.27
3-Octanone	13.71	nd	0.26^{a}	nd	nd	nd	8.80^{b}	nd	nd	nd	nd	0.29^{a}	0.68^{a}	nd	0.04^{a}	nd
2-Butanon-3-Hydroxy	15.73	0.22	0.20	0.74	0.22	nd	0.26	0.22	nd	0.18	nd	0.05	0.17	nd	0.13	0.12
Aceto-phenone	35.46	0.38	0.19	0.18	0.38	0.03	0.11	0.38	nd	0.11	nd	0.09	0.27	nd	0.14	0.15
Phenols		3.82	12.13	11.61	3.82	19.39	41.78	3.82	13.40	35.64	15.52	41.27	65.93	15.52	30.09	58.57
Guaiacol	47.09	nd	nd	nd	nd	3.65^{a}	10.27^{c}	nd	2.24^{a}	4.65^{ab}	3.37^{a}	10.95^{c}	13.47^{c}	3.37^{a}	3.15^{a}	5.21^{b}
Creosol (Homoguaiacol)	51.83	3.74^{a}	11.95^{c}	11.48^{c}	3.74^{a}	14.34^{c}	27.37^{d}	3.74^{a}	10.13^{c}	28.68^{d}	6.92^{b}	20.96^{d}	34.04^{e}	6.92^{b}	24.74^{d}	49.14^{f}
4 Ethyl-guaiacol	55.41	nd	nd	nd	nd	0.22	0.64	nd	0.19	0.50	nd	nd	0.56	nd	0.35	0.63
p-Cresol	58.04	nd	0.10	nd	nd	0.17	0.26	nd	nd	0.30	nd	nd	0.35	nd	0.27	0.32
4-Ethyl-phenol	62.33	nd	nd	nd	nd	0.81^{a}	3.17^{b}	nd	0.49^{a}	1.09^{a}	4.74^{b}	8.48^{c}	17.08^{d}	4.74^{b}	1.36^{a}	3.20^{b}

Table 3.4 Volatile compound area (expressed as percent of total area) of Grossa di Spagna (S) cultivar samples (S1 to S5) detected at 60, 90 and 120 days.

Grossa di Spagna cv		S1			S2			S3			S4			S5		
		Days			Days			Days			Days			Days		
Compounds	RT*	60	90	120	60	90	120	60	90	120	60	90	120	60	90	120
Acids		2.77	1.94	4.51	2.77	50.58	74.09	2.77	46.68	31.01	28.17	66.57	74.40	28.17	24.04	17.50
Acetic acid	25.04	1.24^{b}	1.34^{b}	3.06^{c}	1.24^{b}	1.18^{b}	nd	1.24^{b}	2.76^{c}	0.48^{a}	1.62^{b}	2.75^{c}	0.65^{a}	1.62^{b}	2.36^{c}	0.16^{a}
Propionic acid	30.20	1.53^{a}	nd	0.97^{a}	1.53^{a}	48.84^{c}	73.40^{d}	1.53^{a}	43.42^{c}	30.09^{b}	26.11^{b}	61.90^{d}	73.13^{d}	26.11^{b}	20.96^{b}	16.90^{b}
Isobutyric acid	31.94	nd	0.20	0.24	nd	0.19	0.34	nd	0.30	0.45	0.09	0.14	0.41	0.09	0.34	0.43
Butanoic acid	35.29	nd	0.09	0.25	nd	0.37	0.32	nd	0.20	nd	0.34	1.77^{a}	0.20	0.34	0.37	nd
Alcohol		67.55	38.49	69.52	67.55	11.41	6.92	67.55	12.12	15.01	2.84	5.59	5.36	2.84	18.97	20.93
Ethanol	3.68	62.50^{c}	28.73^{b}	54.31^{c}	62.50^{c}	1.70^{a}	1.34^{a}	62.50^{c}	3.65^{a}	0.28^{a}	nd	3.90^{a}	nd	nd	1.53^{a}	0.48^{a}
Isoamyl-alcohol	12.50	2.68^{b}	5.28^{c}	13.75^{d}	2.68^{b}	1.93^{ab}	0.77^{a}	2.68^{b}	4.04^{c}	5.58^{c}	1.71^{a}	nd	1.74^{a}	1.71^{a}	4.63^{c}	5.35^{c}
Phenyl-ethyl-alcohol	49.80	1.48^{a}	2.03^{ab}	nd	1.48^{a}	6.72	4.07^{b}	1.48^{a}	3.14^{b}	8.37^{c}	0.69^{a}	1.14^{a}	2.81^{ab}	0.69^{a}	11.01^{d}	12.85^{d}
Aldeydes		0.90	3.70	2.24	0.90	2.09	0.39	0.90	1.23	1.20	0.99	1.96	2.13	0.99	2.47	3.37
Octanal	15.34	0.12	0.30	0.16	0.12	0.41	0.16	0.12	0.16	0.18	0.13	0.07	0.23	0.13	0.39	0.88^{b}
Nonanal	21.26	0.42^{a}	1.88^{c}	0.99^{b}	0.42^{a}	0.90^{b}	nd	0.42^{a}	0.52^{a}	0.50^{a}	0.52^{a}	1.21^{b}	1.33^{b}	0.52^{a}	1.20^{b}	1.21^{b}
Decanal	27.40	0.30^{a}	1.08^{b}	0.68^{a}	0.30^{a}	0.61^{a}	nd	0.30^{a}	0.48^{a}	0.41^{a}	0.34^{a}	0.59^{a}	0.37^{a}	0.34^{a}	0.66^{a}	1.03^{b}
Benzaldehyde	28.32	0.05	0.07	0.24	0.05	0.17	0.18	0.05	0.07	0.08	nd	0.09	0.20	nd	0.23	0.13
Esters		24.23	50.98	14.26	24.23	16.97	3.37	24.23	26.48	15.05	64.43	21.31	5.82	64.43	28.24	18.28
Ethyl-acetate	2.97	18.65^{e}	46.89^{f}	10.07^{d}	18.65^{e}	0.82^{a}	0.24^{a}	18.65	2.29^{b}	0.29^{a}	1.66^{b}	4.38^{c}	nd	1.66^{b}	1.53^{b}	nd
Methyl-propanoate	3.15	nd	nd	nd	nd	1.87^{a}	0.62^{a}	nd	3.85^{b}	0.98^{a}	25.89^{c}	nd	0.99^{a}	25.89^{c}	4.20^{b}	1.46^{a}
Ethyl-propanoate	3.89	nd	nd	nd	nd	5.11^{c}	nd	nd	13.67^{d}	2.62^{b}	33.78^{e}	14.20^{d}	2.89^{b}	33.78^{e}	4.73^{c}	0.88^{a}
Isoamyl-acetate	7.92	0.16^{a}	1.02^{b}	0.25^{a}	0.16^{a}	0.07^{a}	nd	0.16^{a}	0.32^{a}	0.21^{a}	nd	1.59^{b}	0.08^{a}	nd	0.89^{b}	nd
Isoamyl-propionate	10.34	nd	nd	nd	nd	nd	nd	nd	1.51^{b}	6.07^{d}	1.29^{b}	nd	0.49^{a}	1.29^{b}	5.24^{d}	3.68^{c}
Ethyl-lactate	19.17	0.80^{a}	1.27^{a}	2.45^{b}	0.80^{a}	1.32^{ab}	0.31^{a}	0.80^{a}	0.83^{a}	0.15^{a}	0.42^{a}	0.80^{a}	0.21^{a}	0.42^{a}	0.56^{a}	0.18^{a}
Ethyl-succinate	37.89	0.17^{a}	0.11^{a}	nd	0.17^{a}	0.32^{a}	nd	0.17^{a}	0.12^{a}	nd	0.07^{a}	nd	nd	0.07^{a}	0.39^{a}	5.36^{b}
Phenyl-ethyl-acetate	44.63	0.08^{a}	0.48^{a}	0.31^{a}	0.08^{a}	0.17^{a}	0.15^{a}	0.08^{a}	0.17^{a}	0.21^{a}	0.21^{a}	0.04^{a}	0.13^{a}	0.21^{a}	1.15^{b}	2.19^{c}
Benzenepropanoic acid ethyl-ester	48.16	3.05^{c}	0.61^{a}	0.39^{a}	3.05^{c}	1.99^{b}	0.73^{a}	3.05^{c}	2.55^{b}	3.46^{c}	0.79^{a}	nd	0.53^{a}	0.79^{a}	5.61^{d}	2.24^{b}
Ketones		0.25	0.45	0.52	0.25	0.16	0.22	0.25	0.15	0.29	0.06	0.15	0.23	0.06	0.13	0.23
2-Butanone-3-hydroxy	15.73	0.25	0.13	0.52	0.25	nd	0.22	0.25	0.08	0.16	0.06	0.15	0.12	0.06	0.01	0.16
Acetophenone	35.46	nd	0.05	nd	nd	0.16	nd	nd	0.07	0.13	nd	nd	0.11	nd	0.12	0.06
Phenols		2.85	3.73	5.64	2.85	18.07	13.44	2.85	12.50	34.36	3.26	4.21	10.41	3.26	25.88	38n81
Creosol (Homoguaiacol)	51.83	2.77^{a}	3.63^{a}	5.48^{b}	2.77^{a}	16.92^{d}	12.55^{c}	2.77^{a}	11.25^{c}	31.81^{f}	2.92^{a}	3.97^{a}	9.44^{c}	2.92^{a}	21.46^{e}	36.70^{f}
4 Ethyl-phenol	62.33	0.04	0.02	0.04	0.04	0.28	0.11	0.04	0.45	0.92	0.06	0.05	0.10	0.06	0.33	0.89

Figure 3.3 PCA plot showing the distribution of table olive samples fromGiarraffa (G1, G5) and Grossa di Spagna (S1, S5) cv.



Sensory results

ANOVA results of sensory analysis (data not shown) reported that the olive samples of the Giarraffa cv were significantly different ($p \leq 0.05$) for the descriptors off-odor and crispy, while ANOVA results of the Grossa di Spagna cv showed significant differences ($p \leq 0.05$) for green color, sour, bitter, green olive flavor and off-flavor descriptors. In Figures 3.4 ad 3.5 are illustrated the sensory profile of the Giarraffa and Grossa di Spagna cv respectively, obtained using the sensory data means. In detail, in the Giarraffa cv, the sample G1 exhibited the lowest intensity of the descriptors off-odor and crispy, while the G2 and G5 samples showed the highest intensity of off-odor and crispy (Figure 3.4). The S1 sample of Grossa di Spagna cv showed the lowest intensity of the descriptors green color, sour, bitter and off-flavor, while showing the highest intensity of green olive flavor. The S2 and S5 samples showed the highest intensity of bitter and off-flavor. The S3 and S5 samples exhibited the lowest intensity of green olive flavor, and the S3 sample showed the highest intensity of sour (Figure 3.5).

Figure 3.4 Sensory profile of table olive samples of Giarraffa cultivar at 120 days of fermentation.

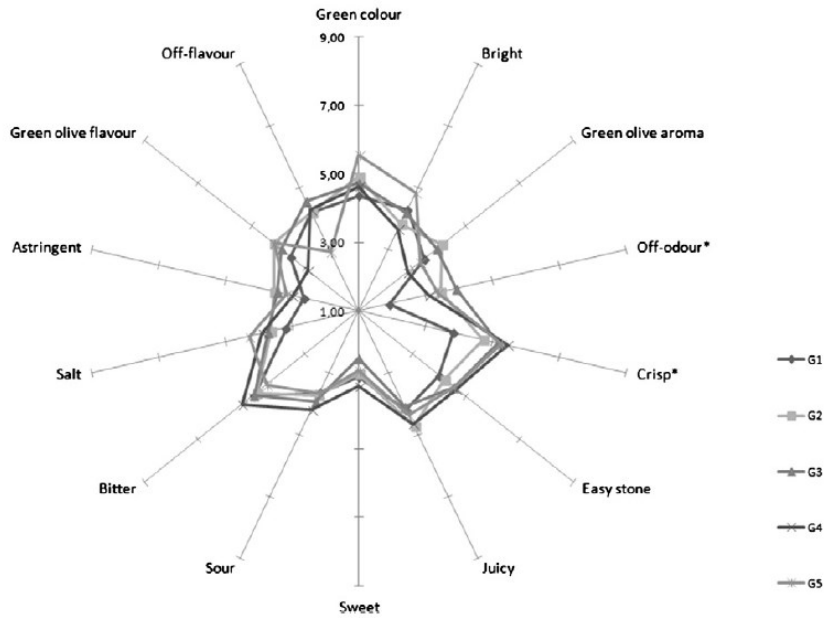
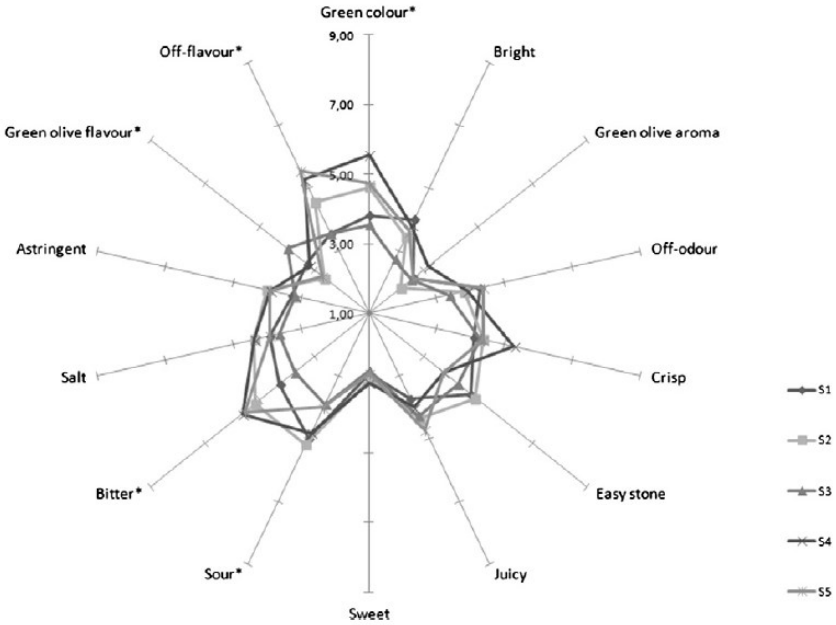


Figure 3.5 Sensory profile of table olive samples of Grossa di Spagna cultivar at 120 days of fermentation.



DISCUSSION

In order to investigate the influence of the microbiota on volatile compounds and on sensory parameters, for the first time, a comparative study between spontaneous and starters inoculated table olives from Giarraffa and Grossa di Spagna cv has been conducted. Moreover, in order to enhance the already important nutritional value of table olive with their potential health-promoting functional features, probiotic strains were deliberately inoculated onto brines. In the last years, fermented foods of plant origin have been increasingly considered as vectors for incorporation of probiotic cultures following the well established procedure of vegetable fermentation (17). Development of probiotic olives may convey a favourable economic impact, especially knowing that such products originate in less developed regions of the EU. In Sicily table olive production is a spontaneous un-controlled process with a complex ecosystem, in which the indigenous microbiota present in the raw material and in the processing environment is the main actor (28, 36-38). Many authors reported that the spontaneous fermentation both of Spanish and Italian table olives is driven by LAB, mainly belonging to *L. plantarum*, *Lactobacillus paraplantarum*, *L. pentosus* and *L. casei* species (1, 21, 30, 37, 49). Nevertheless, the process is not fully predictable and strongly influenced by the competitive activity of yeast population and of a variety of contaminating microorganisms from fermentation vessels and other devices in contact with the olives and brine (1, 7, 19, 31). To prevail over the variability of indigenous microbiota of the raw drupes and to produce high-quality final products, starter cultures of lactobacilli or enterococci have been extensively used in Spanish-style olive fermentation with an improved fermentation process (9, 13, 18). Nevertheless, several parameters, such as external temperature and salt concentration, must be monitored to control microbial groups involved in the process. Several authors

demonstrated that often starter inoculum has low survival due to high salt concentrations of the brine or to low temperatures (8, 20). In the present study table olives were harvested and processed using sterile materials in order to minimize external influences in microbial development and the fermentation was carried at 20–22 °C and in 6% of NaCl to better allow the outgrow of starters. In all samples of both cultivars the pH reached a final value below 4.50, that is considered able to inhibit spoilage and pathogen growth during storage (32). Moreover, the lactic acid concentration exhibited a constant increase during all processes. Enterobacteriaceae displayed very low value at the end of fermentation, except in Grossa di Spagna spontaneous sample, which revealed a load of ca 4.5 log CFU ml−1, in accordance with Tofalo et al. (2012) (49) who recovered in brine values between 2 and 6 log CFU ml−1. The population of LAB and yeasts increased steadily both in spontaneous and in inoculated samples and became the principal member of the microbial association in all processes, as previously reported for directly brined green olives (5, 37, 41). In the present study the mixed starter culture added, constituted by *L. plantarum* and *L. paracasei* species, was well adapted in the brine environment, as demonstrated by their high concentration reached after 14 days of fermentation, maintained almost unchanged throughout the process. According to the multiplex PCR results high occurrence of *L. pentosus* was recovered in spontaneous samples, according to previous studies (2, 6, 48), and *L. plantarum* was the most frequent species found in brine samples where it was deliberately added. This species has been widely isolated from table olive samples (10, 15, 20, 39), highlighting the ability to debitter and ferment table olives (54). Other LAB species were also detected and were ascribed to *L. casei* and *Leuconostoc mesenteroides* species. In a previous study, carried out on Sicilian table olives spontaneously fermented, *L. casei* was the dominant LAB species (38) whereas *Leuc. mesenteroides* was

previously detected by De Bellis et al. (2010) (12) in un-debittered table olives fermented at 4 °C and at low NaCl concentration. As reported by Hurtado et al. (2009), the different species dominance could be explained by the different geographic origin of olives. *L. rhamnosus* is not commonly found in table olives, even if De Bellis et al. (2010) (12) registered a low presence at the 30th day of fermentation in debittered green table olives of Bella di Cerignola cv inoculated with a *L. paracasei* strain. In our study the presence of *L. rhamnosus* at the end of the fermentation is attributed to the artificial addition of probiotics, demonstrating that both the *L. rhamnosus* strains used were able to survive during fermentation, even if their main ecological niche is dairy products (23). Our results also revealed that both inoculated probiotic strains did not host LAB and yeast population, which maintained a high number throughout the process. In naturally fermented olives the polyphenol content of the fruit is one of the factors influencing LAB dynamics (20) and their hydrolysis is correlated to the ability of indigenous LAB to degrade oleuropein. During fermentation, flavor compounds arise from metabolisms of indigenous LAB and yeasts which produce volatile compounds from major fruit constituents through various biochemical pathways that involve specific microbial enzymes (43). Methanol, ethanol, acetic acid, other alcohols and esters produced by alcoholic and heterolactic fermentation are the main compounds found in olive brines (29, 42). Our results demonstrated that spontaneous processes were characterized by high contents of ethanol suggesting an alcoholic fermentation. Ethanol concentration varied greatly among treatments; it is mostly produced by yeast fermentation and, in a lesser extent, by heterolactic fermentation. In spontaneous brines the high content of ethyl-acetate could be correlated to the esterification in aqueous phase of acetic acid with ethanol. It is well known that acetate esters are synthesized by an alcohol-acyltransferase which catalyzes the esterification of

volatile alcohols with acetyl CoA molecules to produce volatile esters and free CoA-SH (44). This compound has been previously reported in green fermented olives (27), as well as in other fermented products (40, 46). Inoculated brine samples of Grossa di Spagna cv showed high contents of propionic acid. Studies have demonstrated that the presence of short-chain fatty acids, mainly propionic and butyric acids, with a concurrent degradation of lactic acid, is related to zapatera spoilage in green olive fermentation (26). In our investigation, the lactic acid concentration increased in all samples: Grossa di Spagna cv samples exhibited higher values than Giarraffa cv. The high presence of propionic acid was confirmed by sensory analysis, in which the sour descriptor of olives was significantly affected by propionic acid. A marked decrease of total ester compounds in the Grossa di Spagna cv samples inoculated with starter cultures rather than in Giarraffa cv samples was inversely correlated to the increase of phenol compounds. Among phenols, a high amount of cresol responsible for off-odors and off-flavors, which is probably due to keto-acid degradation (45), was found in all samples, especially in samples inoculated with starter culture plus probiotic strain. These results correlate with sensory data, demonstrating a more pronounced intensity of the descriptors offodor and off-flavor. On the contrary, in the Giarraffa samples added with starters plus probiotic strains, the decrease of acids and the increase of both alcohol and keton compounds, responsible for herbs, cream and butter flavor, were not detected by the panelists which revealed only high intensities of off-odor and crispy. The addition of starters plus probiotic strains affected also the fermentation of Grossa di Spagna cv, in which high intensities of bitter and off-flavor were revealed by panelists. The bitter sensation could be mainly attributed to the great increase of cresol at the end of fermentation, and not to the incomplete debittering of polyphenols present in the fruit.

CONCLUSION

The present study clearly reports that LAB and yeast population are the main microbial groups involved in brine fermentation of Giarraffa and Grossa di Spagna cv. Comparing fermentation trend in both cultivars it is possible to assert that even if the dominant microbial groups revealed similar enumeration and behavior, notable differences among volatile compounds and sensory characteristics were detected, indicating that cultivar strongly influences the final product. Moreover the findings of the present work accentuate the importance of the starter to guarantee the safety of the table olives and that the starter selection must be done taking into account the olive cultivar. Finally, the present study demonstrated that both the probiotic strains were able to survive during the fermentation of Giarraffa and Grossa di Spagna table olives and that further studies must be carried out in order to estimate the survival rate of the probiotic strains on the olive surface at consumption time.

REFERENCES

1. Abriouel H., Benomar N., Cobo A., Caballero N., Fernández Fuentes M.A., Pérez-Pulido R., Galvez A. (2012). Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiology, 32, 308–316.

2. Abriouel H., Benomar N., Lucas R., Gálvez A. (2011). Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally fermented Aloreña green table olives. International Journal of Food Microbiology, 44, 487–496.

3. Aponte M., Blaiotta G., La Croce F., Mazzaglia A., Farina V., Settanni LMoschetti G. (2012). Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. Food Microbiology, 30, 8–16.

4. Argyri A.A., Zoumpopoulou G., Karatzas K.A.G., Tsakalidou E., Nychas, J.G.E., Panagou E.Z., Tassou C.C. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiology, 33, 282–291.

5. Arroyo-López F.N., Querol A., Bautista-Gallego J., Garrido-Fernández A. (2008). Role of yeasts in table olive production. International Journal of Food Microbiology, 128, 189–196.

6. Bautista-Gallego J., Arroyo-López F.N., Rantsiou K., Jiménez-Díaz R., Garrido-Fernández A., Cocolin L. (2013). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Research International, 5, 135–142.

7. Bevilacqua A., Perricone M., Cannarsi M., Corbo M. R., Sinigaglia M. (2009). Technological and spoiling characteristics of the yeast microflora isolated from Bella di Cerignola table olives. International Journal of Food Science and Technology, 44, 2198–2207.

8. Blana V.A., Grounta A., Tassou C.C., Nychas G.J.E., Panagou E.Z. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. Food Microbiology, 38, 208–218.

9. Chorianopoulos N.G., Boziaris I.S., Stamatiou A., Nychas G.J.E. (2005). Microbial association and acidity development of unheated and pasteurized green-table olives fermented using glucose or sucrose supplements at various levels. Food Microbiology, 22, 117–124.

10. Cocolin L., Alessandria V., Botta C., Gorra R., De Filippis F., Ercolini D., Rantsiou K. (2013). NaOH-debittering induces changes in bacterial ecology during table olives fermentation. PLoS ONE, 8, 1–12.

11. Corsetti A., Perpetuini G., Schirone M., Tofalo R., Suzzi G. (2012). Application of starter cultures to table olive fermentation: an overview on the experimental studies. Frontiers in Microbiology, 3, 1–6.

12. De Bellis P., Valerio F., Sisto A., Lonigro S.L., Lavermicocca P. (2010). Probiotic table olives: Microbial populations adhering on olive surface in fermentation sets inoculate with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. International Journal of Food Microbiology, 140, 6–13.

13. De Castro A., Montaño A., Casado F. -J., Sánchez H., Rejano L. (2002). Utilization of *Enterococcus casselinflavus* and *Lactobacillus pentosus* as starter cultures for Spanish style green olive fermentation. Food Microbiology, 19, 637–644.

14. De los Reyes-Gavilan C. G., Limsowtin G. K. Y., Tailliez P., Séchaud L., Acholas J. P. (1992). A *Lactobacillus helveticus*-specific DNA probe detects restriction fragment length polymorphisms in this species. Applied and Environmental Microbiology, 58, 3429–3432.

15. Delgado A., Brito D., Fevereiro P., Marques J.F. (2001). Antimicrobial activity of *L. plantarum*, isolated from a traditional lactic acid fermentation of table olives. Le Lait, 81, 203–215.

16. Garrido-Fernández A., Fernández Díez M.J., Adams M.R. (1997). Table olives, production and processing. London: Champman and Hall.

17. Gupta S., Abu-Ghannam N. (2012). Probiotic fermentation of plant based products: Possibilities and opportunities. Critical Review in Food Science and Nutrition, 52, 183–199.

18. Holzapfel W.H. (1997). Use of starter cultures in fermentation on a household scale. Food Control, 8, 241–258.

19. Hurtado A., Reguant C., Bordons A., Rozès N. (2009). Influence of fruit ripeness and salt concentration on the microbial processing of Arbequina table olives. Food Microbiology, 26, 827–833.

20. Hurtado A., Reguant C., Bordons A., Rozès N. (2012). Lactic acid bacteria from fermented table olives. Food Microbiology, 31, 1–8.

21. Hurtado A., Reguant C., Esteve-Zarzoso B., Bordons A., Rozès N. (2008). Microbial population dynamics during the processing of Arbequina table olives. Food Research International, 41, 738–744.

22. IOC, International Olive Council (2013). Key figures on the work market for table olives. 100th session. 19–23 November 2012. Madrid, Spain. Available on line at: www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures (Accessed on January 2014)

23. Lavermicocca P., Valerio F., Lonigro S.L., De Angelis M., Morelli L., Callegari M.L., Rizzallo C.G., Visconti A. (2005). Study of adhesion and survival of Lactobacilli and Bifidobacteria on table olives with the aim of formulating a new probiotic food. Applied and Environmental Microbiology, 71, 4233–4240.

24. Malheiro R. Guedes, de Pinho P., Casal S., Bent A., Pereira J.A. (2011). Determination of the volatile profile of stoned table olives from different varieties by using HS-SPME and GC/IT-MS. Journal of Science and Food Agriculture, 91, 1693–1701.

25. McFeeters R.F. (2004). Fermentation microorganisms and flavor changes in fermented foods. Journal of Food Science, 69, 35–37.

26. Montaño A., de Castro A., Rejano L., Sánchez A.H. (1992). Analysis of Zapatera olives by gas and high-performance liquid chromatography. Journal of Chromatography, 594, 259–267.

27. Montaño A., Sánchez A. H., De Castro A. (2000). Changes in the amino acid composition of green olive brine due to fermentation by pure culture of bacteria. Food Microbiology and Safety, 65, 1022–1027.

28. Muccilli S., Caggia C., Randazzo C. L., Restuccia C. (2011). Yeast dynamics during the fermentation of brined green olives treated in the field with kaolin and Bordeaux mixture to control the olive fruit fly. International Journal of Food Microbiology, 148, 15–22.

29. Panagou E.Z., Tassou C.C. (2006). Changes in volatile compounds and related biochemical profile during controlled fermentation of cv. Conservolea green olives. Food Microbiology, 23, 738–746.

30. Panagou E.Z., Schillinger U., Franz C., Nychas G.J.E. (2008). Microbiological and biochemical profile of cv. Conservolea naturally black olives during controlled fermentation with selected strains of lactic acid bacteria. Food Microbiology, 25, 348–358.

31. Panagou E.Z., Tassou C.C., Katsaboxakis C.Z. (2003). Induced lactic acid fermentation of untreated green olives of the Conservolea cultivar by *Lactobacillus pentosus*. Journal of the Science of Food and Agriculture, 83, 667–674.

32. Perricone M., Bevilacqua A., Corbo M.R., Sinigaglia M. (2010). Use of *Lactobacillus plantarum* and glucose to control the fermentation of “Bella di Cerignola” table olives, a traditional variety of Apulian region (southern Italy). Journal of Food Science, 75, 430–436.

33. Pitino I., Randazzo C.L., Cross K.L., Parker M.L., Bisignano C., Wickham M.S., Mandalari C., Caggia C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. Food Microbiology, 31, 57–63.

34. Pitino I., Randazzo C.L., Mandalari G., Lo Curto A., Faulks R.M., Le Marc Y., Bisignano C., Caggia C., Wickham M.S. (2010). Survival of *Lactobacillus rhamnosus* strains in the upper gastro-intestinal tract. Food Microbiology, 27, 1121–1127.

35. Pitino I., Randazzo C.L., Restuccia C., Caggia C. (2009). Il Pecorino tradizionale come fonte di lattobacilli probiotici. Scienza e Tecnica Lattiero-Casearia, 60, 179–185.

36. Randazzo C.L., Fava G., Tomaselli F., Romeo F.V., Pennino G., Vitello E., Caggia C. (2011). Effect of kaolin and copper based products and of starter cultures on green table olive fermentation. Food Microbiology, 28, 910–919.

37. Randazzo C.L., Rajendram R., Caggia C. (2010). Lactic acid bacteria in table olive fermentation. In V. R. Pretty, & R. R. Watson (Eds.), Olives and Olive Oil in Health and Disease Prevention, 41. (pp. 371–379). UK: Elsevier978-012-374420-3.

38. Randazzo C.L., Restuccia C., Romano A.D., Caggia C. (2004). *Lactobacillus casei,* dominant species in naturally fermented Sicilian green olives. International Journal of Food Microbiology, 90, 9–14.

39. Randazzo C.L., Ribbera A., Pitino I., Romeo F.V., Caggia C. (2012). Diversity of bacterial population of table olives assessed by PCR-DGGE analysis. Food Microbiology, 32, 87–96.

40. Roza C., Laca A., Garcia L. A., Díaz M. (2003). Ethanol and ethyl acetate production during the cider fermentation from laboratory to industrial scale. Process Biochemistry, 38, 1451–1456.

41. Ruiz-Barba J.L., Cathcart D.P., Warner P.J., Jiménez-Díaz R. (1994). Use of Lactobacillus plantarum LPCO10, a bacteriocin producer, as a starter culture in

Spanish style green olive fermentations. Applied and Environmental Microbiology, 60, 2059–2064.

42. Sabatini N., Mucciarella M.R., Marsilio V. (2008). Volatile compounds in un-inoculated and inoculated table olives with Lactobacillus plantarum (Olea europaea L., cv. Moresca and Kalamata). Food Science and Technology, 41, 2017–2022.

43. Sabatini N., Perri E., Marsilio V. (2009). An investigation on molecular partition of aroma compounds in fruit matrix and brine medium of fermented table olives. Innovative Food Science and Emerging Technologies, 10, 621–626.

44. Salas J.J. (2004). Characterization of alcohol acyltransferase from olive fruit. Journal of Agricultural and Food Chemistry, 52, 3155–3158.

45. Smit G., Smit B.A., Engels W.J. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiology Reviews, 29, 591–610.

46. Soufleros E.H., Mygdalia S.A., Natskoulis P. (2005). Production process and characterization of the traditional Greek fruit distillate “Koumaro” by aromatic and mineral composition. Journal of Food Composition and Analysis, 18, 699–716.

47. Tassou C.C., Panagou E.Z., Katsaboxakis K.Z. (2002). Microbiological and physicochemical changes of naturally black olives fermented at different temperatures and NaCl levels in the brines. Food Microbiology, 19, 605–615.

48. Tofalo R., Perpetuini G., Schirone M., Fasoli G., Suzzi G., Corsetti A. (2014). Lactobacillus pentosus dominates spontaneous fermentation of Italian table olives. LWT—Food Science and Technology, http://dx.doi.org/10.1016/j.lwt.2014.01.35.

49. Tofalo R., Schirone M., Perpetuini G., Angelozzi G., Suzzi G., Corsetti A. (2012). Microbiological and chemical profiles of naturally fermented table olives and brines from different Italian cultivars. Antonie van Leeuwenhoek, 102, 121–131.

50. Torriani S., Felis G.E., Dellaglio F. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *rec*A gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Applied and Environmental Microbiology, 67, 3450–3454.

51. UNAPROL (2009). Scenario economico di settore. Available at: http://www.unaprol.it/ (accessed the 2nd of March, 2012)

52. UNI 10957 (2003). Sensory analysis—Method for establishing a sensory profile in foodstuffs and beverages. Milano, Italia: UNI: Ente Nazionale Italiano di Unificazione.

53. Ventura M., Canchaya C., Meylan V., Klaenhammer T.R., Zink R. (2003). Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. Applied and Environmental Microbiology, 69, 6908–6922.

54. Zago M., Lanza B., Rossetti L., Muzzalupo I., Carminati D., Giraffa G. (2013). Selection of *Lactobacillus plantarum* strains to use as starters in fermented table olives: Oleuropeinase activity and phage sensitivity. Food Microbiology, 34, 81–87.

Chapter 4

Lactobacillus rhamnosus GG for Bacterial Vaginosis treatment

Alessandra Pino, Cinzia Lucia Randazzo, Antonio Cianci, Emanuela Bartolo, Cinzia Caggia.

Manuscript for publication in preparation

GENERAL OVERVIEW

THE VAGINAL MICROBIOTA COMPOSITION IN HEALTH AND IN DISEASE

The female genital tract is composed of a sequence of cavities and it is exposes to the external environment with the risk of potential infections. Among the defence mechanisms, one of the most important is the composition of the microbial communities that colonize the vagina (76). The microbial inhabitants of the vagina constitute a finely balanced ecosystem with the vaginal environment controlling the colonizing bacteria and the microbiota, in turn, controlling the vaginal environment. This dynamic microbial community plays a key role in preventing colonization by undesirable microrganisms.

Much of our knowledge, about the composition of the vaginal microbial communities comes from qualitative and semi-quantitative descriptive studies using cultivation-dependent techniques (37, 43, 69). In recent years, the introduction and the development of cultivation-independent molecular-based techniques have revolutionized bacterial detection and provided new information about the microbial phylogenetic diversity that compose the vaginal environment. This ecosystem has been revealed considerably very dynamic and complex comprising previously undetected bacterial vaginal inhabitants (74, 79, 25). The advent of next-generation ultra-high-throughput sequencing technologies has removed an important quantitative barrier in molecular analysis by increasing the number of reads from a gene or genome (63). The recent transition from low-throughput clone library sequencing studies to deep sequencing of PCR amplicons has led to a rapid accumulation of data regarding human associated microbial communities and has been crucial in furthering our understanding of the microbiota colonizing the genital tract (55). The most detailed investigation to date used pyrosequencing of barcoded 16S rRNA gene

to probe the vaginal microbiota (VM) in healthy women of childbearing age. According to that, the current knowledges suggest that the vaginal ecosystem of healthy women typically shows a predominance of *Lactobacillus* species. In particular, the strains most frequent are *Lactobacillus crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* (52, 71), followed by *L. rhamnosus*, *L. reuteri*, *L. vaginalis*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum* and *L. salivarius* (53, 20, 6-8). The healthy VM does not contain high numbers of many different species of *Lactobacillus*. Rather, a single species among the most frequent or a group of closely related species are dominant, whereas other species are rare, lower in cell density, and tend to be novel phylotypes (72, 75, 80, 12, 67, 78). The rare coexistence of multiple species of lactobacilli in vaginal communities could be caused by competitive exclusion of one species by another, pre-emptive colonization by a particular species or host factors that strongly influence which species are able to colonize the vaginal environment (79). Interestingly, apparently healthy vaginal ecosystem is maintained in a significant proportion (7–33%) of women in the absence of a *Lactobacillus*-dominant VM (36, 7, 8). Lactobacilli may be replaced by other lactic acid-producing bacteria, such as *Atopobium vaginae*, *Megasphaera* spp., and *Leptotrichia* spp. Moreover, a diverse array of other bacteria and microorganisms, such as *Staphylococcus*, *Ureaplasma*, *Corynebacterium*, *Streptococcus*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Mycoplasma*, *Enterococcus*, *Escherichia*, *Veillonella*, *Bifidobacterium* and *Candida* (36) can be present but in much lower amounts. Lactobacilli are involved in promoting a healthy vaginal environment by preventing overgrowth of pathogenic and opportunistic organisms. This mechanism of action is attributable to lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins productions (61). On the other hand, *Lactobacillus*-deficient VM is associated with the development of numerous infectious conditions such as bacterial vaginosis (BV). Oral or vaginal antibiotics

administration is the traditionally BV treatment and in this regard metronidazole and vaginal clindamycin (1, 2) are frequently used. Treatment with antimicrobial agents is useful but when is stopped, BV associated bacteria quickly re-emerge, suggesting a possible role for intermittent prophylactic treatment. Alternative cures are therefore desirable. In this contest, there are important issues to which great attention must be paid regarding the effects of probiotics on BV treatment and prevention. Probiotics are believed to protect the host against infections by several mechanisms including the following: occupation of specific adhesion sites at the epithelial surface of the urinary tract; maintenance of a low pH and production of antimicrobial substances like acids, hydrogen peroxide, and bacteriocins; degradation of polyamines; production of surfactants with antiadhesive properties (7, 27). As reported in table 4.1 several clinical trials were conducted in order to investigate the usefulness of probiotic administration for BV treatment and prevention. These trials confirmed the ability of lactobacilli to restore and maintain a normal urogenital flora and showed that probiotic bacteria, especially *L. acidophilus*, *L. rhamnosus* GR-1, and *L. fermentum* RC-14, when administered over 10^8 CFU, can most appropriately normalize the VM, can help cure the existing infection and can prevent recurrence of BV. Longer periods of probiotic administration may be useful for long-term control of BV relapses after conventional therapy with metronidazole. Probiotics have been reported useful when used either vaginally or orally (34, 18, 57); foods and supplements have both been shown to be efficient vehicles as well (35).

Table 4.1. Effects of administration of probiotics on BV (performed between 1990 and 2011).

Intervention	Heath condition	Effects	Reference
Oral yoghurt containing $1.0x10^8$ CFU of *L. acidophilus*, once daily for 2 months	Bacterial vaginosis, Candidiasis	Reduction in BV episodes at 1 months was 60% for probiotic yoghurt vs 25% for pasteurized	65
Oral capsules containing 10^8 CFU of *L. rhamnosus* GR-1 plus *L. fermentum* RC-14 or *L. rhamnosus*, each day for 28 d	Bacterial vaginosis	Normal vaginal flora was restored using specific probiotic strains administered orally	58
Skim milk containing 10^9 CFU of *L. rhamnosus* GR-1 and *L. fermentum* RC-14, twice daily for 14 days	Bacterial vaginosis	Treatment correlated with a healthy vaginal flora in up to 90% of patients	26
Oral capsule containing $8x10^8$ CFU of *L. rhamnosus* GR-1/*L. fermentum RC-14* and $6x\ 10^9$ CFU *L. rhamnosus* GR-1/*L. fermentum* RC-14 3-*L. rhamnosus* GR-1/*L. fermentum* RC-14, daily for 28 d	History of BV	Through 6 weeks after treatment with probiotics, Nugent score decreased, indicative of BV resolution	6
Oral capsule containing $9x10^9$ CFU of *L. rhamnosus* GR-1 + *L. fermentum* RC-14, once daily for 60 d	Bacterial vaginosis	Probiotics colonized the vagina properly and the Nugent score normalized after the treatment	58
Oral capsules containing 10^9 CFU of *L. rhamnosus* GR-1 and *L. fermentum* RC-14, 60 days	Urogenital infections	Lactobacilli counts increased while yeast and coliforms decreased significantly after supplementation	57

Intervention	Heath condition	Effects	Reference
Oral capsule containing 10^9 CFU of *L. reuteri* RC-14, *L. rhamnosus* GR-1, twice daily from Days 1 to 30	Bacterial vaginosis	88% were cured in the antibiotic/probiotic group compared to 40% in the antibiotic/placebo group. High counts of *Lactobacillus* spp. colonized the vagina properly	7
Oral capsules containing $2.5x10^9$ CFU of *L. rhamnosus* GR-1 and *L. reuteri* RC-14, 14 days	Bacterial vaginosis	The median difference in Nugent scores between baseline and the end of the study was 3 in the intervention group and 0 in the control group	54
Vaginal tampons containing 10^8 CFU of *L. gasseri*, *L. casei* var *rhamnosus*, and *L. fermentum*,5 tampons during menstruation	Bacterial vaginosis	Microbiological cure was observed based on Nugent score and Amsel criteria	24
Vaginal tablet containing $9x10^7$ CFU of *L. acidophilus*, 0.03 mg of estriol and 600 mg of lactose, daily for 6 d	Vaginal infections	Vaginal flora was enhanced significantly by the probiotic administration in combination with low-dose estriol	50
Vaginal tablet containing 10^4 CFU of *L. rhamnosus*, once a week at bedtime for 2 months	Bacterial vaginosis	Significant difference between the 2 treatment groups were seen at Day 90	41
Vaginal tablets containing 10^9 CFU of *L. brevis*, *L. salivarius* subsp. *salicinius*, and *L. plantarum*, for 7 days	Bacterial vaginosis	All of the patients in the probiotic group were free of BV, showing a normal or intermediate vaginal flora	46

Intervention	Heath condition	Effects	Reference
Vaginal application containing 10^4 CFU *of L. rhamnosus*, for 6 months	Prevent the recurrence of bacterial vaginosis	The vaginal administration of the probiotic allows stabilization of the vaginal flora and reduces BV recurrence	42
Vaginal capsules containing between 10^8 and 10^{10} CFU of *L. gasseri* LN40, *L. fermentum* LN99, *L. casei* subsp. *rhamnosus* LN113, and *P. acidilactici* LN23, for 5 d, after conventional treatment of bacterial vaginosis	Bacterial vaginosis, vulvovaginal candidiasis	LN had a good colonization rate in the vagina of patients with BV and women receiving LN were cured 2Y3 days after administration	23
Vaginal capsule containing 10^8 CFU of *L. rhamnosus*, *L. acidophilus*, and *S. thermophilus*	Prophylaxis bacterial vaginosis	Probiotic prophylaxis resulted in lower recurrence rates for BV women	77

Effect of *Lactobacillu rhamnosus* GG and Lactoferrin administration on vaginal microbiota of women with bacterial vaginosis.

INTRODUCTION

The most familiar condition associated to the vaginal microbiota (VM) imbalance is bacterial vaginosis (BV), which affect about the 10-29% of women in the word (15, 4). BV is a polymicrobial disorder, whose etiology has not been fully understood. It involves with the replacement of normal vaginal hydrogen peroxide-producing lactobacilli by a variety of gram-negative rods and mycoplasmas. Microbiologically, lactobacilli are reduced, absent or lacking specific antimicrobial properties (i.e. production of H_2O_2) with the concurrent high concentration of numerous other opportunistic pathogenic bacteria mainly *Gardnerella vaginalis* but also other anaerobes such as *Atopobium vaginae*, *Bacteroides*, *Mobiluncus*, *Prevotella*, *Peptostreptococcus* spp., *Ureaplasma urealyticum* and *Mycoplasma hominis* (17, 46). It is not clear whether the primary event triggering BV is the loss of key lactobacilli or the acquisition of a complex bacterial community typically found in this syndrome. Current treatment strategies for BV include the oral or intra-vaginal administration of metronidazole or clindamycin. The use of oral metronidazole for 7 days or vaginal metronidazole for 5 days implicate an improvement of symptoms in 83% to 87% of women within 2 to 3 weeks (40, 30). Similar response rates are observed with the use of vaginal clindamycin. Vaginal recolonization rates with lactobacilli are similar with both antibiotics, as defined by detection of lactobacilli on Gram stain 21 to 30 days after initiation of antibiotic treatment (2, 49). Even though antibiotic medication is effective in up to 90% of cases, about 25% of women will develop BV again within 4 weeks (31, 39) and the long-term recurrence rates have been shown to be >70% (63, 69, 14). Moreover, there

are several unpleasant side effects and disadvantages associated with antibiotic therapies including infections with pathogenic microorganisms, susceptibility of lactobacilli to clindamycin and increase of drug resistance showed by vaginal phatogens, particularly *G. vaginalis* and anaerobic bacteria (11, 69). Because of its high morbidity and frequent recurrence following antibiotic treatment, alternative therapeutic agents need to be sought for the treatment of BV. In this contest, probiotic and Lactoferrin administration can represent a promising alternative treatment. There are important issues to which great attention must be paid regarding the effects of probiotics on BV treatment and prevention. Several clinical trials confirmed the ability of probiotics to treat BV by restoring the VM balance and preventing the relapses. Lactoferrin (Lf) is an approximately 80-kDa iron-binding glycoprotein belonging to the transferrin family produced and stored in specific (secondary) neutrophil granules and released during neutrophil activation and degranulation. Lf owns bacteriostatic and bactericidal properties, with the ability to protect the host against infection, by binding and regulating the iron needed for the bacterial proliferation (61, 9, 74). The antimicrobial effect of Lf is also due to immunomodulation as well as cytoplasmic membrane disruption of the target cell. Lf has an inhibitory effect on LPS-induced production of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8 mRNA) and in monocyte cells interfering with NF-κB activation (19, 38, 82). Additional functions of Lf have been reported, such as neutrophil and macrophage activation (67, 28), regulation of specialization and function of lymphocytes (28), activation of the natural killer cells (21), and control of the oxidation injury (71). Moreover, Lf exhibits a synergistic effect with IgA, lysozyme, antibiotics, and drugs which helps in eradication of microorganisms (65). Based on the properties previously reported is possible to postulate that lactoferrin could be able to re-establish the vaginal microbial balance in BV patients but up to now no study have

been conducted in this regard. Moreover, even if the usefulness of probiotic administration, both on BV treatment and prevention, is largely demonstrated no study are available concerning the effectiveness of *L. rhamnosus* GG (LGG) and lactoferrin administration. In the light of the evidence above, this study was aimed to evaluate if the lactoferrin administration alone or in combination with LGG is able (i) to modify the composition and dynamics of VM in BV and (ii) to restore the VM homeostasis condition.

MATERIALS AND METHODS

Patients enrolment

The present study was an open prospective randomized controlled trial. From October 2013 to November 2014 40 female patients, aged between 18 and 45 years, with BV signs and symptoms were enrolled at the Department of Medical Surgery Speciality, University Hospital G. Rodolico, Catania, Italy. Eligible subjects were sexually active women in reproductive age with symptomatic acute BV. The diagnosis of BV was assessed using the Amsel clinical criteria (5) and confirmed using Gram stain criteria (Nugent scores) (48). The exclusion criteria included: Nugent score <7, considered as the threshold of a significant imbalance in the vaginal microbiota composition; urogenital infections or sexual transmitted disease; known seropositivity for the human immunodeficiency virus (HIV); pregnancy or breastfeeding; antibiotic, probiotic, exogenous hormone treatments and other gynaecological conditions that could cause bleeding (polyps, endometrial hyperplasia, etc.). The study was conducted in accordance with the Helsinki Declaration (2000) of the World Medical Association and current standards of good clinical practice.

The local Ethics Committee approved the trial and informed written consent was obtained from all participants prior to enrolment.

Study design

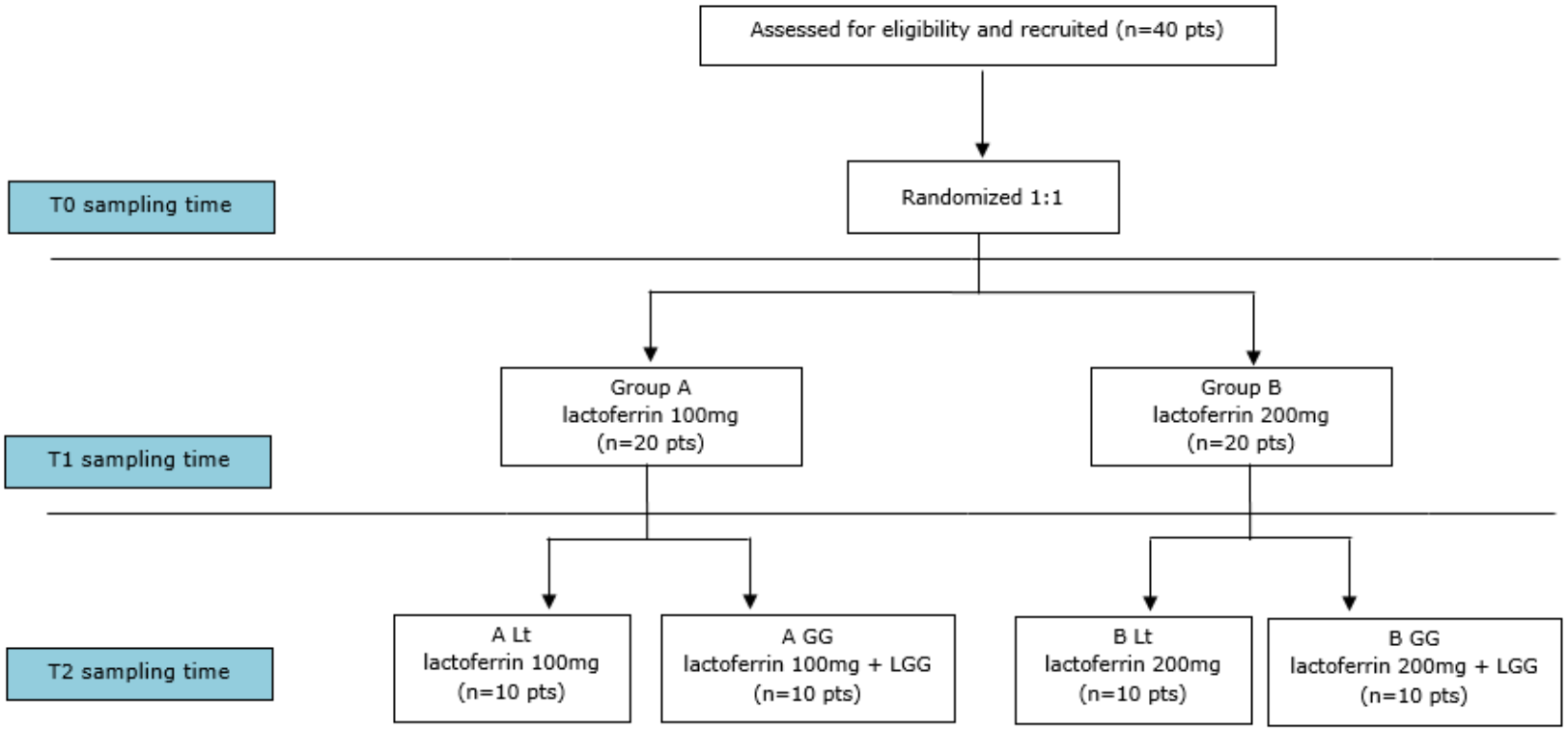
Patients satisfied the inclusion criteria previously reported, were recruited and randomized in two groups (A and B) and were treated with Lf 100mg (group A, n=20) or Lf 200mg (group B, n=20) for 10 days (Figure 4.1). The sample size of 20 subjects per group was based on the expectation of finding a difference of 50% between the two groups. At the first follow-up visit (T1) the patients allocated in each group were split in two subgroups and subjected to different treatment for additional 10 days (T2) as followed reported. Subgroups derived from group A were treated with Lf 100 mg alone (A Lt) or in combination with LGG (A GG); while subgroups derived from group B, were treated with Lf 200 mg alone (B Lt) or in combination with LGG (B GG). Therapy was initiated after the enrolment visit or immediately after the end of menstruation if menses were expected within a 7-day period. Lf was administered as vaginal tablets and each female subject was instructed to apply the tablet once a day preferably before going to bed. LGG (10^9 UFC) was taken orally once a day. Patients were not allowed to use others probiotics, topic or orally, as well as antibiotic agents for two months previous the recruitment and at any time during the study. All patients completed the study without dropouts. The study was divided in three times of intervention: baseline (T0), 10 days Lf treatment (T1) and 10 days after the additional administration of Lf or Lf in combination with LGG (T2). Demographics and medical information concerning contraceptive habits, infectious disease history, sexual activity and menstrual period were assessed at baseline. Pelvic examination, assessment of clinical

signs and symptoms of BV and microbiological sampling were performed at each time of intervention.

Sample collection

Vaginal swab specimens collected during each sampling time (T0, T1 and T2) were subjected both to microbiological analysis and to total DNA extraction. In addition, two cotton-tipped swabs were rolled over glass slides and used to assess Nugent score and whiff-amine/sniff test in accordance with Amsel's criteria. Vaginal fluid pH was measured during each visit by using pH test strips (McKesson). Samples were collected at the Department of Medical Surgery Speciality, University Hospital G. Rodolico, Catania, Italy, and immediately transferred, under refrigeration condition, to the Laboratory of Microbiology of D3A University of Catania, Italy.

Figure 4.1 Flow diagram: enrolment and follow-up diagram of patients included in the study.



Clinical criteria evaluation

The Nugent score was assessed on a 10-point scale performing Gram-stain of the vaginal glass smears followed by optical microscopic observation under oil immersion (100X magnification). Large gram-positive bacilli were assumed to be the *Lactobacillus* morphotype; smaller gram-variable bacilli were assumed to be the *Gardnerella* morphotype; other organisms were categorized by morphology only, e.g., gram-negative bacilli, curved rods, gram-positive cocci in chains and fusiforms. A score of 0-3 was interpreted as normal microbiota, a score of 4-6 was assumed as intermediate and a score of 7-10 was considered as BV-like condition. Microscopic observation of vaginal glass smears was also performed in order to detect the presence of "clue-cells". Moreover, according to Amsel's criteria, the whiff-amine/sniff test was carried out. In detail, a drop of 10% KOH was directly added to the glass surface and the presence of fish odour, attributable to volatile amines production, was revealed with sense of smell. Clinical criteria previously reported were evaluated during each sampling time and all slides were analysed by the same investigator.

Microbiological analysis

Vaginal swabs collected by using a sterile synthetic swab tip Transystem Amies Medium Clear (Biolife Srl) were analysed as follow. After dislodging the cells in sterile PBS, serial 1:10 dilutions were made and plated on the following agar media and conditions: Rogosa SL agar (Biolife), incubated at 35-37°C in 5% CO_2 and 95% H_2 for 40-48 hours for *Lactobacillus* counts; de Man-Rogosa-Sharp with Twin 80 (Biolife), anaerobically incubated at 37 °C for 48 h for LAB counts, Streptococcus Selective Agar (Biolife) for isolation, enumeration and identification of

Streptococci, incubated at 32°C for 24 hours; *Gardnerella vaginalis* Selective Medium (Oxoid) incubated at 36±1°C for 40-48 hours, for *Gardnerella vaginalis* isolation; MacConkey Agar Mug (Biolife) incubated at 37° C for 16-18 hours for *Escherichia coli*; Mannitol Salt Agar (Oxoid) incubated at 32 °C for 48 h, for staphylococci enumeration; Slanetz Bartley Agar (Biolife) for Enterococci enumeration, incubated at 37°C for 48 hours; Chromogenic Candida Agar (Biolife) for *C.albicans*, *C.tropicalis*, *C.krusei* enumeration, incubated at 35°C-37°C for 48 h. All analysis were performed in duplicate.

DNA extraction

Genomic DNA was extracted from vaginal swab specimens collected at each sampling time. The bacterial cells retrieved on swabs were submerged in 1 ml of sterile normal saline (prepared with RNase free H2O, pH 7.0) and vigorously agitated to dislodge cells. The cells were pelleted by centrifugation (Thermo Electron Corporation, Boston, MA, USA) at full speed (≥ 10,000 g) for 10 min, washed by re-suspending cells in sterile normal saline and centrifuged at full speed for 5 min. Then, bacterial DNA was extracted from the vaginal swabs using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions with minor modification. Briefly, the bacterial pellet was suspended in 180 µl of lysis buffer (buffer ATL) and homogenized by vortexing. A total of 20 µl of proteinase K solution (20 mg/ml) and 100 mg of zirconium beads (0.1 mm) were then added. The mixture was agitated in a mini bead beater (FastPrep, Thermo Electron Corporation, USA) three times, 40 s each time, and incubated at 56°C for approximately 40 min. 200 µl of the second lysis buffer (buffer AL) provided in the kit was added, and the sample was incubated at 70°C for 10 min. Next, 200 µl of ethanol was added; this mixture was then loaded on the QIAamp spin column and

centrifuged at 8,000 g for 1 min. The QIAamp spin column was placed in a new 2 ml collection microtube, and the containing filtrate was discarded. The column material was washed with 500 µl buffer AW1 and with 500 µl buffer AW2 provided in the kit. Finally, the DNA was eluted with 20 µl of distilled water (2 × 10 µl). The concentration of extracted DNA was determined by using a NanoDrop 2000, Thermo Fisher Scientific (Wilmington, DE, USA); its integrity and size were checked by 1.0% agarose gel electrophoresis containing GelRed Nucleic Acid Gel Stain (Biotium). All DNA was stored at -20°C before further analysis.

Ion Torrent 16S rRNA Gene-Based Analysis

DNA amplification and Ion Torrent PGM Sequencing of 16S rRNA gene-based amplicons were performed by GENPROBIO srl (Parma). DNA extracted from brine samples at initial, middle and end of fermentation and was amplified using primer pair Probio_Uni and/Probio_Rev, which targets the V3 region of the 16S rRNA gene sequence (47). DNA was amplified under the PCR conditions described previously (47). PCR amplicons were analyzed by electrophoresis on an Experion workstation (BioRad, UK) and quantified by using the Experion system (BioRad). Emulsion PCR was performed by using Ion OneTouchTM 200 Template Kit v2 DL (Life Technologies) according to the manufacturer's instructions. Sequencing analyses was carried out according to the protocol of the Ion Torrent PGM system and using the Ion Sequencing 200 kit. Sequence reads were analyzed by PGM software to delete low quality and polyclonal sequences. High quality sequences were trimmed and filtered with default settings, using the QIIME pipeline version 1.4.0 (http://qiime.sourceforge.net). Filtered sequences were exported as sff files.

Taxonomic identification

The sff sequence files were processed using QIIME (3). The sequences were first clustered into Operational Taxonomic Unit (OTU) clusters with 97% identity (3% divergence).

All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the Ribosomal Database Project (16). OTUs were assigned using uclust (22). Alpha diversity (rarefaction, Good's coverage, Chao1 richness and Shannon diversity indices) and beta diversity measures were calculated and plotted using QIIME. Final datasets at species and other relevant taxonomy levels were compiled into separate worksheets for compositional analysis among the samples. Differences in microbial communities between brines samples were also investigated using using UPGMA clustering (Unweighted Pair Group Method with Arithmetic mean) on the distance matrix of OTU abundance. This resulted in a Newick formatted tree, which was obtained by using QIIME package (47).

Statistical analysis

The analysis of variance (ANOVA) was carried out on transformed data followed by separation of means with Tukey's HSD, using the statistical software Statistica for Windows (Statistica 6.0 per Windows 1998, StatSoft, Vigonza, Italy). Letters indicate significant different groups ($P<0.05$) by Tukey's test.

RESULTS

Clinical criteria

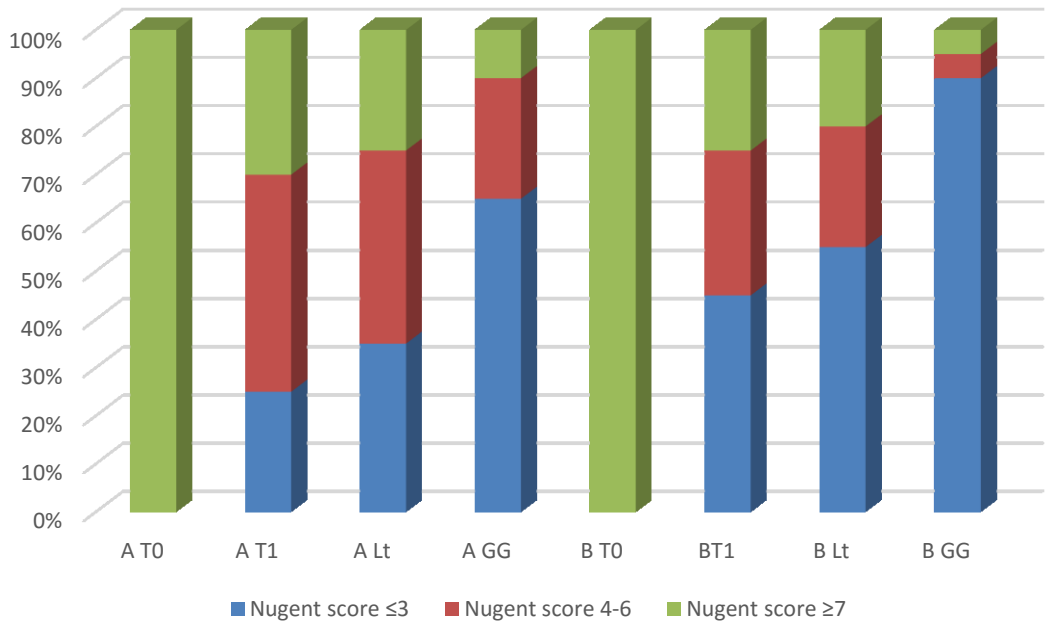
The Nugent score, the Sniff test percentage of positivity and the vaginal pH value are reported in table 4.2. Moreover, in figure 4.2 are reported the occurrence (%) of women with normal, intermediate or bacterial vaginosis evaluated by Nugent score. Both groups A and B showed at baseline the Nugent score value ≥7 confirming the presence of BV. In group A the Lf 100 mg administration for 10 days (T1) reduced the Nugent score below the threshold of 7 in 14 patients of 20 (70%). In detail, five patients (25%) showed a Nugent score < 3 (healthy status), nine (45%) presented an intermediate microbiota (Nugent score between 4 and 6) whereas the remaining six patients (30%) showed a score >7 considered as BV-like condition. The Lf 100mg administration for additional 10 days (subgroup A Lt) was responsible of few changes in Nugent score while the administration of Lf 100mg in association with LGG (subgroup A GG) exhibited significant increase of number of patients with Nugent score <3. In fact, in the latter subgroup only 2 patients presented the Nugent score >7 (10%), five (25%) were characterized by intermediate microbiota (score between 4 and 6) and thirteen (65%) showed Nugent score <3 at T2 sampling time. Concerning group B, the Lf 200 mg administration for 10 days (T1) induced the reduction of Nugent score below the threshold of 7 in fifteen patients. Six (30%) of these presented Nugent score between 4 and 6 while nine (45%) showed normal vaginal microbiota composition (score <3). At T2 sampling time the Nugent score shifts observed were more pronounced in B GG subgroup (Lf 200 mg and LGG administration) then in B Lt one (LT 200mg administration). In detail, at the end of the intervention the 55% of patients, allocated in subgroup B Lt, showed normal vaginal microbiota while the 90% of patients assigned to B GG resulted to be BV negative. During the study, the decrease of number of patients with

Nugent score > 7 was in accordance with the wiff/sniff test percentage of positivity that decreased reaching the lower value in both A GG and B GG subgroups. Similar trend was detected for the number of patients that showed vaginal pH value > 4.5.

Table 4.2 Clinical criteria of both A and B groups during the whole of the trial.

Group	Time of intervention	Nugent score ≤3	Nugent score 4-6	Nugent score ≥7	sniff test positivity	Vaginal pH >4.5
A	T0	0	0	20	90%	19
	T1	5	9	6	32%	7
A Lt	T2	7	8	5	25%	6
A GG	T2	13	5	2	15%	3
B	T0	0	0	20	92%	20
	T1	9	6	5	30%	6
B Lt	T2	11	5	4	16%	4
B GG	T2	18	1	1	5%	1

Figure 4.2 Occurrence (%) of women with vaginal microbiota normal, intermediate or bacterial vaginosis evaluated by Nugent score.



Microbial counts

Microbial counts, expressed as average and standard deviation of log CFU/mL, and analysis of variance of the main microbial groups detected in A and B groups during the study, were reported in table 4.3. Both groups showed at baseline (T0) a complex microbiota composition in accordance to BV condition. All microbial groups investigated were detected at high cell density except for Lactobacilli and Lactic acid bacteria (LAB). In group A, the Lf 100 mg administration for 10 days (T1) determined the decrease of *Gardnerella vaginalis* of about 1 log cycle (p=0.000). Enterococci exhibited similar trend to those depicted from *Gardnerella vaginalis* with significant decrease in cell density (p=0.004). As expected, Lactobacilli and LAB

showed significant increase with an average of 0.8 log cycles higher than baseline. No significant change was detected for *Staphylococcus* spp., *Streptococcus* spp., *E*. *coli*, and *Candida* spp. counts. The administration of Lf 100mg for additional 10 days (A Lf subgroup, T2 sampling time) was able to determine significant change of all microbial groups analysed, except for *E. coli*, *Lactobacillus* spp., and LAB. Differently, the administration of Lf 100mg in addition to LGG for 10 days (A GG subgroup, T2 sampling time) determined significant change in all microbial groups. In particular, *Gardnerella vaginalis* shifted from 1.76 log CFU/ml (T1) to 0.78 log CFU/ml (p=0.000), *Lactobacillus* spp. and Lactic acid bacteria increase of about 1 log cycle reaching a final values of 5.90 log CFU/ml and 6.03 log CFU/ml respectively. Group B showed at T1 sampling time (Lf 200mg administration for 10 days) significant change in all microbial groups except for *Enterococcus* spp. and *Candida* spp. In particular, *Gardnerella vaginalis* decreased of about 1 log cycle (p=0.000) and *Lactobacillus* spp. increase of about 1.5 log cycle reaching a value of 5.54 log CFU/ml. Subgroup B Lt showed no significant change of *Lactobacillus* spp. (p=0.181) and Lactic acid bacteria (p=0.169) compared to T1 sampling time. Significant change was instead detected after the administration of Lf 200mg in addition to LGG (B GG subgroup, T2 sampling time). In fact, all microbial groups showed significant change with p value equal to 0.000. In particular, *Gardnerella vaginalis* reaching a value of 0.34 log CFU/ml, *Lactobacillus* spp. and Lactic acid bacteria increased an average of about 1.40 log cycle compared to T1 sampling time.

Table 4.3 Microbial counts, standard deviation (SD) and Anova significance of A and B groups during the intervention.

Microbial groups	Microbial counts (log UFC/ml) and SD													
	GROUP A							GROUP B						
	T0	T1	T2		P	P	P	T0	T1	T2		P	P	P
			A Lf*	A GG**	T0vsT1	T1vsT2*	T1vsT2**			B Lf*	B GG**	T0vsT1	T1vsT2*	T1vsT2**
Staphilococcus spp.	2.16±0.05	1.96±0.05	1.49±0.07	1.03±0.05	0.165	0.001	0.000	3.42±0.02	2.87±0.04	2.16±0.02	1.18±0.03	0.030	0.004	0.000
G. vaginalis	2.44±0.05	1.76±0.04	1.46±0.06	0.78±0.03	0.000	0.026	0.000	3.37±0.03	2.43±0.05	1.47±0.02	0.34±0.05	0.000	0.000	0.000
Streptococcus spp.	3.41±0.04	3.40±0.05	2.99±0.08	2.58±0.06	0.465	0.002	0.002	4.59±0.08	4.06±0.06	2.90±0.03	1.76±0.03	0.015	0.000	0.000
Enterococcus spp.	3.52±0.07	3.17±0.05	2.84±0.05	2.84±0.04	0.004	0.016	0.016	4.50±0.03	4.44±0.02	3.25±0.03	2.05±0.04	0.398	0.000	0.000
E.coli	0.39±0.03	0.41±0.04	0.29±0.03	0.03±0.02	0.423	0.084	0.000	1.02±0.08	0.61±0.05	0.37±0.08	0.07±0.05	0.047	0.002	0.000
Candida	0.07±0.01	0.02±0.00	0.03±0.00	0.03±0.05	0.072	ND	ND	0.47±0.04	0.43±0.06	0.21±0.03	0.03±0.02	0.329	0.053	0.000
Lactobacillus spp.	4.08±0.12	4.86±0.05	4.72±0.10	5.90±0.04	0.000	0.205	0.000	3.95±0.02	5.54±0.02	5.72±0.02	6.90±0.04	0.000	0.181	0.000
LAB	4.11±0.06	4.95±0.05	4.95±0.05	6.03±0.03	0.000	0.491	0.000	4.47±0.02	6.10±0.05	6.30±0.04	7.51±0.04	0.000	0.169	0.000

* Lt administration for additional 10 days; ** Lt and GG administration for additional 10 days

Ion Torrent 16S rRNA gene-based analysis

The DNA extracted from vaginal swab specimens collected during each sampling time (T0, T1, T2) was subjected to Ion Torrent 16S rRNA gene-based analysis. A mean value of approximately 59.500 reads was analyzed, with the highest value (93579) detected at T1 sampling time in B group and the lowest value (39945) founded at the same sampling time in A group (Table 4.4). The bacterial community was analyzed using rarefaction curves, a species-level measure (OTU), a richness estimator (Chao1) and a Shannon diversity index (Table 4.4). The biodiversity observed and the estimated sample coverage (Good's coverage values about 99%) for the 16S rDNA vaginal swab samples indicated that a satisfactory coverage of the biodiversity was achieved. This result was also confirmed by the analysis of the rarefaction curves (Figure 4.3). The OTU, Chao1, and Shannon index values showed opposite trend in A and B groups comparing T0 and T1 sampling time. In fact, these parameters increased in A group and decreased in B group. At T2 sampling time OTU, Chao1, and Shannon index values decreased in all subgroups (A Lt, A GG, B Lt, B GG). The highest OUT value were achieved in A group after the Lf 100mg administration for 10 days (T1) while in B group at baseline (T0). Principal coordinate analysis (PCoA), using unweighted UniFrac distance matrix (Figure 4.5), was done and allowed us to differentiate the effect of different treatments. In detail, it was possible to distinguish 3 clusters. The first one comprised both A and B groups at baseline (T0) in addition to A Lt (Lf 100 mg administration for 20 days in the whole), which was quite diverse from the cluster 2 that included the following groups: B T1 (after 10 days of Lf 200 mg administration), B Lt (after 20days of Lf 200 mg administration), and A T1 (after 10 days of Lf 100 mg administration). The patients subjected to LGG administration in addition with Lf 100 mg (A GG group) or 200 mg (B GG group) were grouped in the cluster 3. The vaginal microbiota

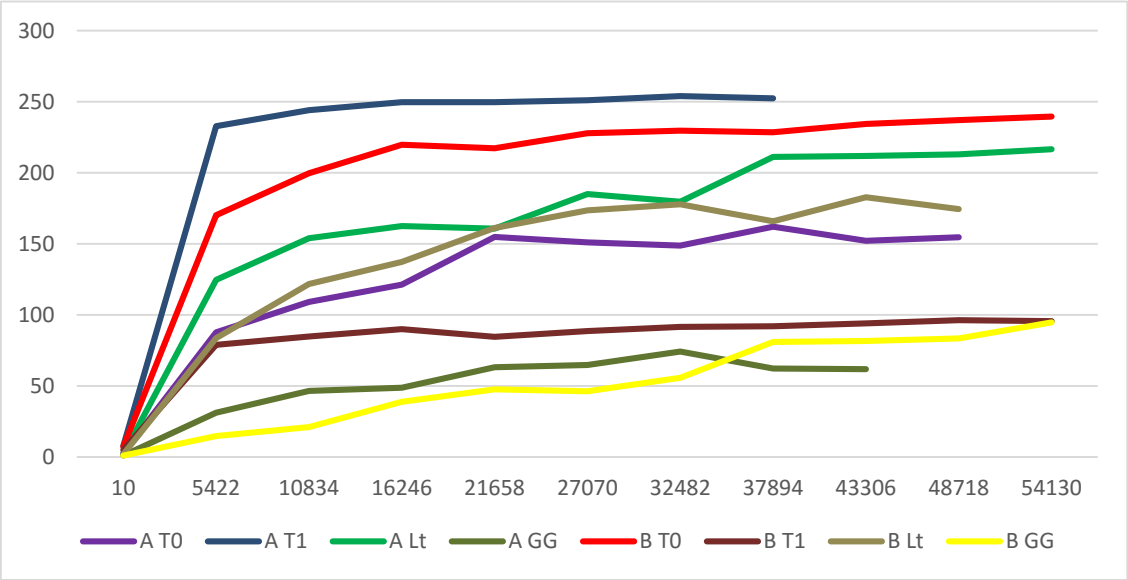
composition of each group at phylum, family and genus level are reported in figures 4.5, 4.6 and 4.7 respectively. In detail, group A was characterized at baseline (T0) by the dominance of *Firmicutes* phylum (82.90%) followed by *Actinobacteria* (16.40%), mainly belonged to *Gardnerella* genera (16.25%). The phylum previously reported was dominant also in group B at T0 sampling time, in addition to *Proteobacteria* phylum (7.07%), mainly belonged to *Acinetobacter* (4.00%) and *Helicobacter* (1.57%). Significant shifts in bacterial community were observed in group A in particular after the administration of Lf 100mg in addition to LGG. In fact, *Firmicutes* (99.84%) was the only dominant pylum represented by *Lactobacillus* genera (99.79%). The dominance of *Firmicutes* phylum and *Lactobacillus* genera was achieved in group B not only during the administration of Lf 200mg in addition to LGG (B GG) but also during the Lf 200mg administration for additional 10 days (B Lt). At genus and species level, each groups revealed a unique species-specific profile, even the presence of *L. helveticus* species was achieved in all patients after the LGG administration.

Table 4.4 Number of sequences analyzed, biodiversity measures and estimated sample coverage (%) of total 16S rRNA gene of vaginal swab specimens.

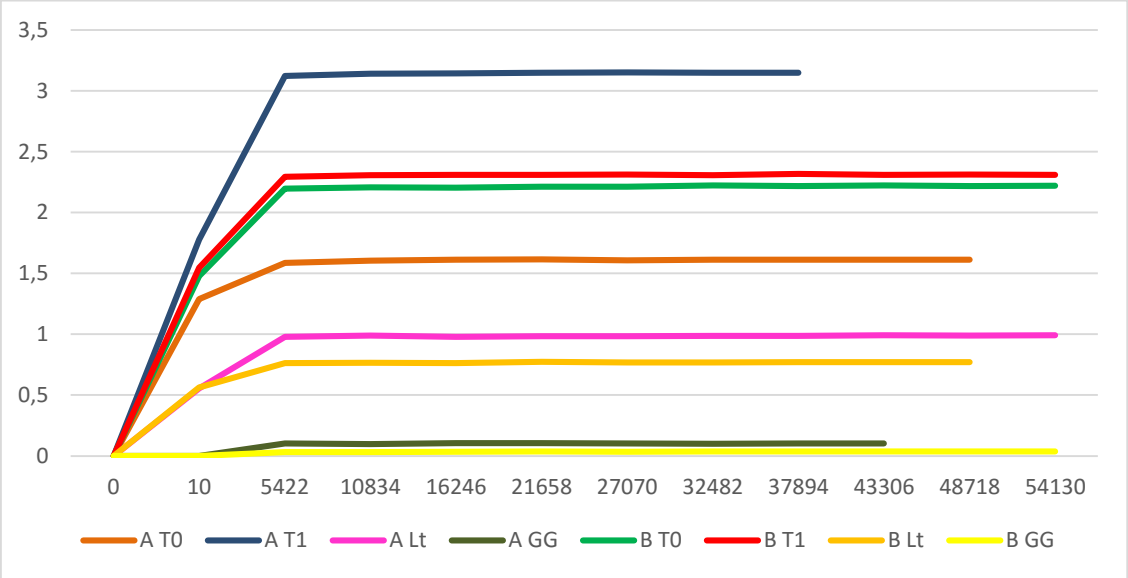
	GROUP A				GROUP B			
	T0	T1	T2		T0	T1	T2	
			A Lt	A GG			B Lt	B GG
N° reads	51350	39945	71098	45336	55223	93579	53048	66510
OTUs	34	105	30	7	78	49	17	5
Chao 1	2.9	7.7	2	1	7.2	4.9	1.9	1
Shannon index	1.28	1.77	0.55	0	1.47	1.55	0.56	0
Good's coverage (%)	99.4	98.9	99.1	99.8	99.2	99.5	99.8	99.7

Figure 4.3 Rarefaction curves generated for 16S rRNA gene sequences obtained from the samples.

a)

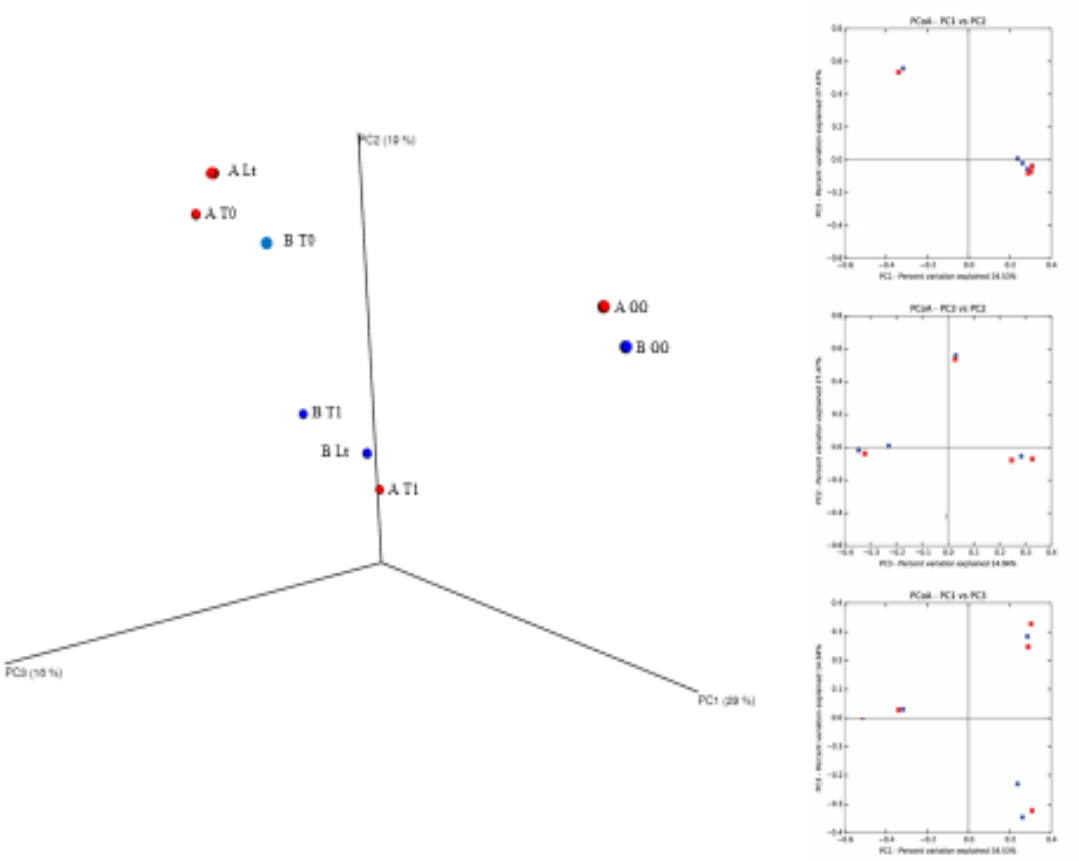


b)



Panel a represents the rarefaction curves using the Chao1 index. Panel b display rarefaction curves using the Shannon index.

Figure 4.4 Principal Coordinate Analysis (PCoA).



Percentages shown along the axes represent the proportion of dissimilarities captured by the axes.

Figure 4.5 Phylum-level distribution of bacteria in vaginal samples, taxa >0.1% are shown.

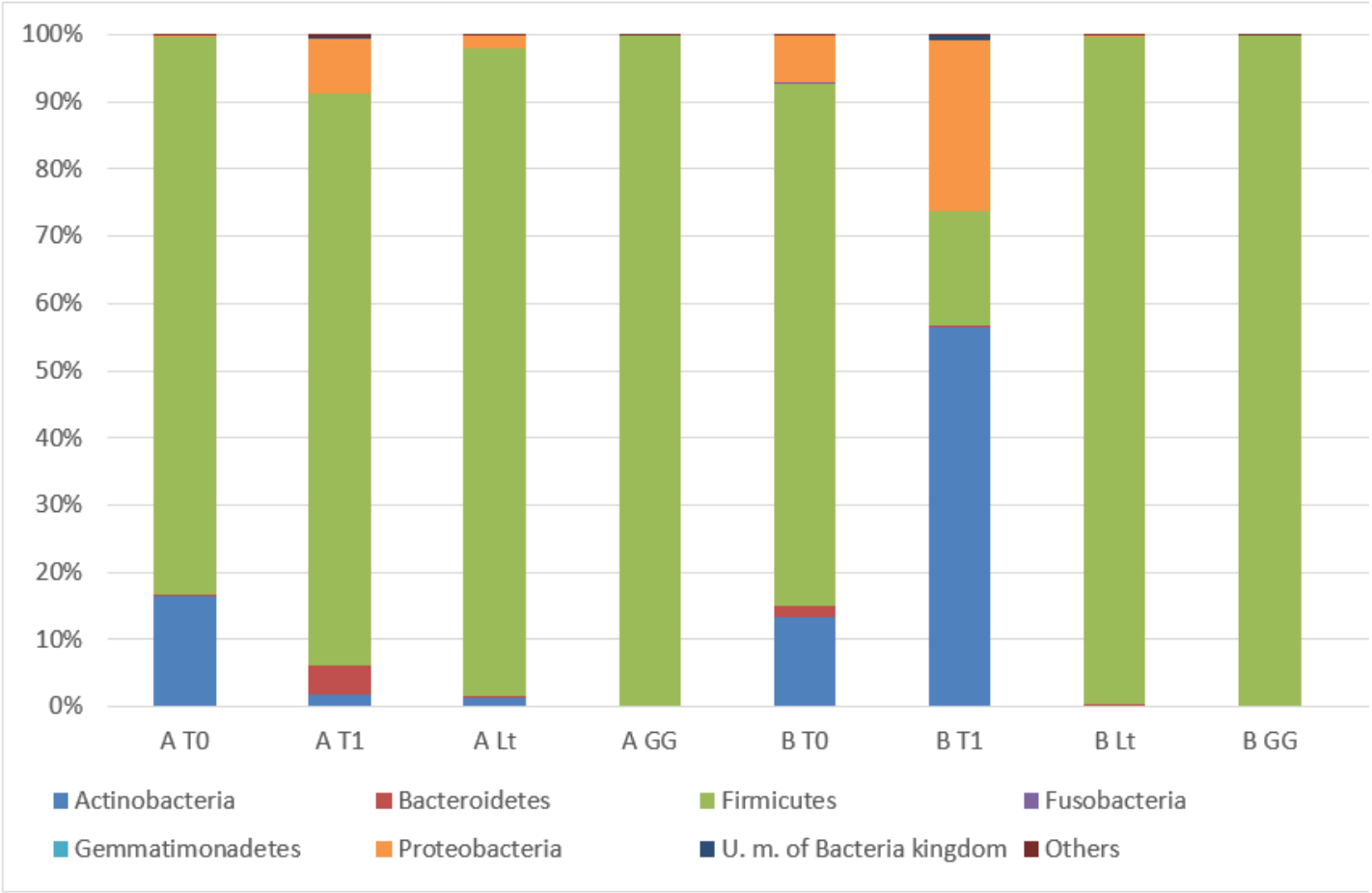


Figure 4.6 Family-level distribution of bacteria in vaginal samples, taxa >0.1% are shown.

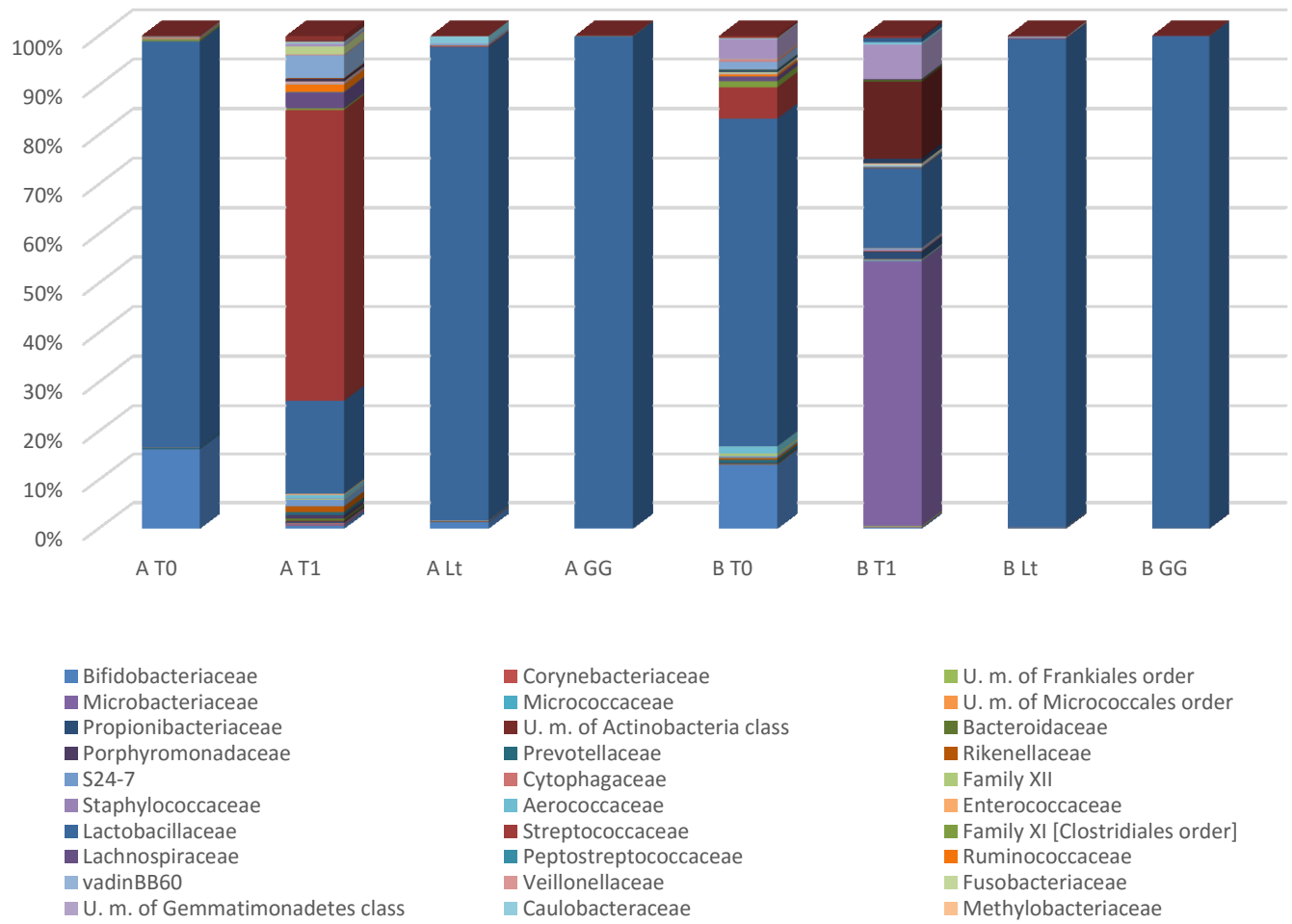
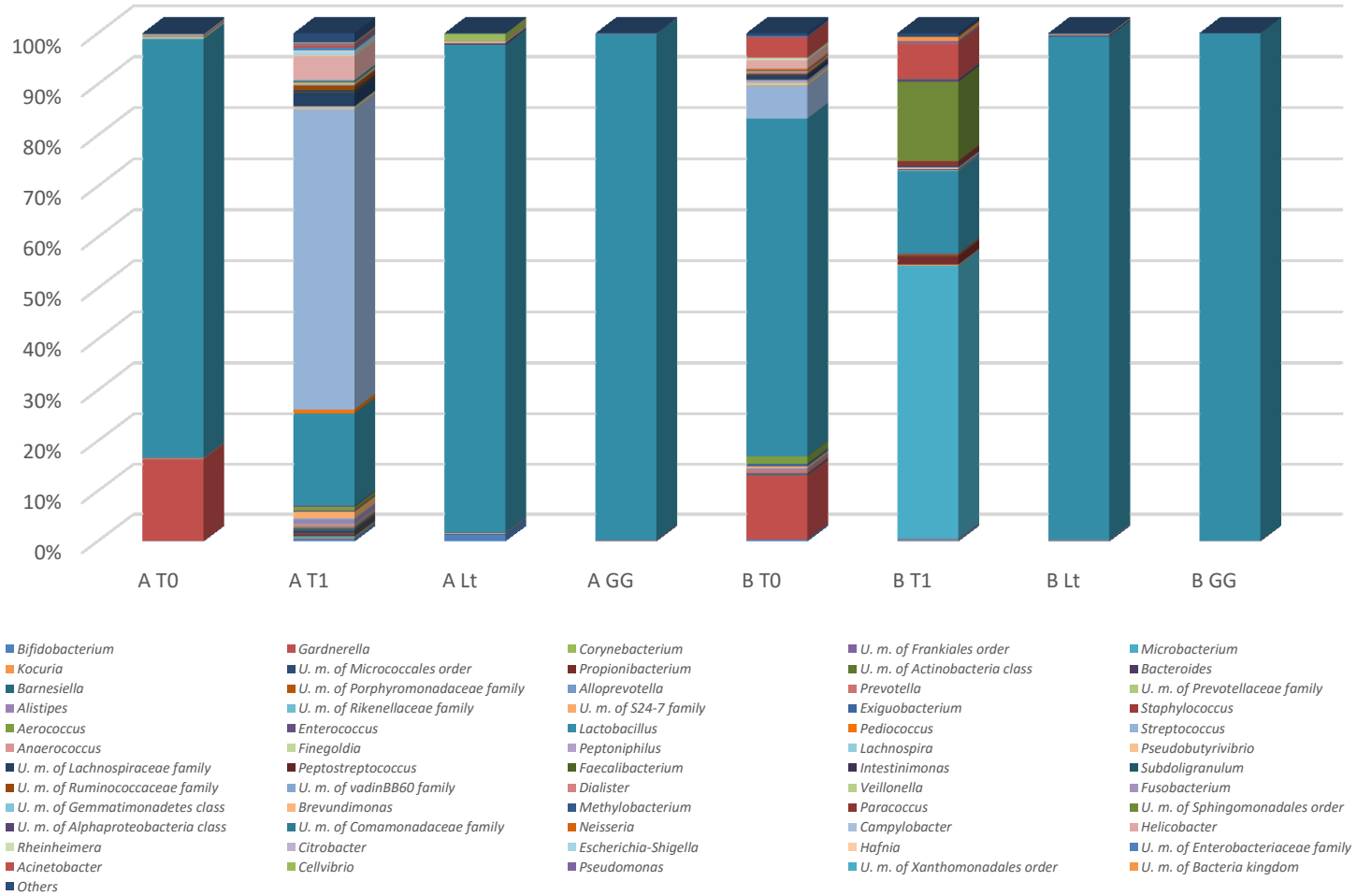


Figure 4.7 Genus-level distribution of bacteria in vaginal samples, taxa >0.1% are shown.



DISCUSSION

It is already well established that vaginal microbiota consist predominantly of lactobacilli and that alterations can cause symptomatic conditions (33). Comprehensive knowledge of the composition and richness of the vaginal microbial ecosystem is essential for understanding the etiology of diverse vaginal diseases, for its prevention and for the development of new diagnostic tools. In this contest, bacterial vaginosis is an ecological disorder affected millions of women annually, associated with numerous adverse health outcomes including pre-term birth and the acquisition of sexually transmitted infections. Treatment with antibiotic agents is useful but when is stopped, BV associated bacteria quickly re-emerge, suggesting a possible role for intermittent prophylactic treatment. For these reasons, an alternative treatment is desirable. According to Mazzaro et al (2006) (44), given the high prevalence of BV, there is an urgent need to develop products that effectively treat this condition and prevent its recurrence. Studies of the effects of probiotics on bacterial vaginosis are relatively scarce. The lactobacilli strains most frequently used are a combination of *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14. *In vitro* studies demonstrated their ability to adhere to vaginal epithelium cells (13) and to colonize the vagina also when are orally administrated (29). Anukam et al. (6) conducted a randomized, controlled, double-blind study confirming the positive effects of combined use of metronidazole with capsules containing probiotic in bacterial vaginosis treatment. After the 30-day period, 88% of patients who received probiotic had no signs of bacterial vaginosis, in comparison to 40% vaginosis-free patients in placebo group ($p < 0.001$). The same authors also conducted another randomized clinical trial, which compared the efficacy of probiotic vaginal capsules used with the metronidazole gel in treatment of bacterial vaginosis (7), with comparable results. Similar results were published by Martinez et al. (45)

(combination of tinidazole and probiotic in comparison with tinidazole and placebo), and by Ya et al. (77) who recently conducted a randomized, double-blind, placebo-controlled study testing the effectiveness of probiotic vaginal capsules in preventing recurrences of bacterial vaginosis. Although not without certain shortcomings in terms of population size, inclusion conditions or follow up methodology, the previously mentioned studies established a strong case for the existence of beneficiary effect of Lactobacilli in subjects with bacterial vaginosis. To our knowledge, this is the first study on the ability of lactoferrin administration alone or in combination with LGG to treat bacterial vaginosis. Our findings confirm that LGG administration in association with lactoferrin both at 100mg and at 200 mg is able to modify the vaginal microbiota population and to re-establish the balance of this ecosystem. These evidences suggested by clinical criteria evaluation and microbiological results, was supported by Ion Torrent 16S rRNA Gene-Based results. Not only after 20 days all patients presented the vaginal microbiota dominated by lactobacilli but it was interesting to found the dominance of L. *helveticus* particularly in patients treated with LGG and lactoferrin 200mg. As is known *L. helveticus* is considered a strain with health-promoting properties that possess probiotic properties similar to those displayed by microorganisms conventionally considered to be probiotics. Atassi et al. 2006 (10) demonstrated its ability to inhibit the growth and to reduce the viability of *Gardnerella vaginalis* and *Prevotella bivia*. Based on our results, the LGG and lactoferrin administration can represent a valid treatment for bacterial vaginosis. Moreover, we found that clinical criteria evaluation is essential as diagnostic tool but to better understand the effect of a specific therapeutic treatment microbiological and molecular analysis are essential. The culture-dependent and independent approach and the Ion Torrent

16S rRNA Gene-Based results were able to provide a more detailed view of vaginal microbial composition and dynamic during the trial.

REFERENCES

1. ACOG Committee on Practice Bulletins-Gynecology. ACOG Practice Bulletin. Clinical management guidelines for obstetrician-gynecologists. (2006). Obstetrics & Gynecology, 107,1195-1206.
2. Agnew and Hillier JM,McGregor JA, Hillier SL, Eschenbach D.A., Kreutner A.K., Galask R.P., Martens M. (2000). Metronidazole for bacterial vaginosis: a comparison of vaginal gel vs. oral therapy. Journal of Reproductive Medicine, 45, 889–896.
3. Albers S.V., Meyer B.H. (2011). The archaeal cell envelope. Nature Reviews Microbiology, 9, 414–426.
4. Allsworth J.E., Peipert J.F. (2007). Prevalence of bacterial vaginosis: 2001–2004 National Health and Nutrition Examination Survey data. Obstetrics & Gynecology, 109, 114–120.
5. Amsel R., Totten P.A., Spiegel C.A., Chen K.C., Eschenbach D., Holmes K.K. (1983). Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. The American Journal of Medicine, 74, 14–22.
6. Anukam K., Osazuwa E., Ahonkhai I., Ngwu M., Osemene G., Bruce A.W., Reid G. (2006). Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR1 and *Lactobacillus reuteri* RC14: randomized double blind placebo controlled trial. Microbes and Infection, 8, 1450–1454.
7. Anukam K., Osazuwa E., Osemene G.I., Ehigiagbe F., Bruce A.W., Reid G. (2006). Clinical study comparing probiotic *Lactobacillus* GR-1 and RC-14 with metronidazole

vaginal gel to treat symptomatic bacterial vaginosis. Microbes and Infection, 8, 2772–2776.

8. Anukam K.C., Osazuwa E.O., Ahonkhai I., Reid G. (2006). Lactobacillus vaginal microbiota of women attending a reproductive health care service in Benin city, Nigeri". Sexually Transmitted Diseases, 33, 59–62.

9. Arnold R.R., Brewer M., Gauthier J.J. (1980). Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. Infection and Immunity, 28, 893-898.

10. Atassi F., Brassart D.,Grob P.,Graf F., Servin A.L. (2006). In vitro antibacterial activity of *Lactobacillus helveticus* strain KS300 against diarrhoeagenic, uropathogenic and vaginosis associated bacteria. Journal of applied microbiology, 101, 647–654.

11. Beigi R.H., Austin M.N., Meyn L.A., Krohn M.A., Hillier S.L. (2004). Antimicrobial resistance associated with the treatment of bacterial vaginosis. American Journal of Obstetrics & Gynecology, 191, 1124–1129.

12. Biagi E., Vitali B., Pugliese C., Candela M., Donders G.G., Brigidi P. (2008). Quantitative variations in the vaginal bacterial population associated with asymptomatic infections: a real-time polymerase chain reaction study. European Journal of Clinical Microbiology & Infectious Diseases, 28, 281-285.

13. Boris S., Suarez J., Vazquez F., Barbés C. (1998). Adherence of human vaginal Lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infection and Immunity, 66, 1985–1989.

14. Bradshaw C.S., Morton A.N., Hocking J., Garland S.M., Morris M.B., Moss L.M., Horvath L.B., Kuzevska I., Fairley C.K. (2006). High recurrence rates of bacterial

vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. Journal of Infectious Disease, 193, 1478–1486.

15. Cauci S., Driussi S., De Santo D. Penacchioni P., Iannicelli T., Lanzafame P., De Seta F., Quadrifoglio F., de Aloysio D., Guaschino S. (2002). Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. Journal of Clinical Investigation, 40, 2147–2152.

16. Cole J.R., Wang Q., Cardenas E., Fish J., Chai B., Farris R.J., Kulam-Syed-Mohideen A.S., McGarrell D.M., Marsh T., Garrity G.M., Tiedje J.M. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research, 37, 141–145.

17. Coste I., Judlin P., Lepargneur J.P., Sami Bou A. (2012). Safety and efficacy of an intravaginal prebiotic gel in the prevention of recurrent bacterial vaginosis: a randomized double-blind study. Obstetrics and Gynecology International, 1-7, Article ID 147867.

18. Cribby S., Taylor M., Reid G. (2008). Vaginal microbiota and the use of probiotics. Interdisciplinary Perspectives on Infectious Diseases, 1-9, Article ID 256490.

19. Crouch S.P.M., Slater K.J., Fletcher J. (1992). Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. Blood, 80, 235-240.

20. Damelin L.H., Paximadis M., Mavri-Damelin D., Birkhead M., Lewis DA., Tiemessen CT. (2011). Identification of predominant culturable vaginal Lactobacillus species and associated bacteriophages from women with and without vaginal discharge syndrome in South Africa. Journal of Medical Microbiology, 60, 180–183.

21. Damiens E., Mazurier J., El Yazidi I., Masson M., Duthille I., Spik G., Boily-Marer Y. (1998). Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1402, 277–287.

22. Edgar R.C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 26, 2460–2461.

23. Ehrström S., Daroczy K., Rylander E., Samuelsson C., Johannesson U., Anzén B., Påhlson C. (2010). Lactic acid bacteria colonization and clinical outcome after probiotic supplementation in conventionally treated bacterial vaginosis and vulvovaginal candidiasis. Microbes and Infection, 12, 691-699.

24. Eriksson K., Carlsson B., Forsum U., Larsson P.G. (2005). A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open treatment with vaginal clindamycin ovules. Acta Dermato-Venereologica, 85, 42-46.

25. Fredricks D.N., Fiedler T.L., Marrazzo J.M. (2005). Molecular identification of bacteria associated with bacterial vaginosis. The New England Journal of Medicine, 353, 1899-1911.

26. Gardiner G.E., Heinemann C., Baroja M.L., Bruce A.W., Beuerman D., Madrenas J., Reid G. (2002). Oral administration of the probiotic combination *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 for human intestinal applications. International Dairy Journal, 12, 191-196.

27. Goldin B.R., Gorbach S.L. (2008). Clinical indications for probiotics: an overview. Clinical Infectious Diseases, 46, 96-100.

28. Gonzalo de la Rosa, Yang D., Tewary P., Varadhachary A., Oppenheim J.J. (2008). Lactoferrin acts as an alarmin to promote the recruitment and activation of antigen-presenting cells and antigen-specific immune responses. Journal of Immunology, 180, 6868-6876.

29. Hager W.D., Rapp R.P. (1992). Metronidazole. Obstetrics and Gynecology Clinics of North America, 19, 497–510.

30. Hanson J.M, McGregor J.A., Hillier S.L., Eschenbach D.A., Kreutner A.K., Galask R.P., Martens M. (2000). Metronidazole for bacterial vaginosis: a comparison of vaginal gel vs. oral therapy. Journal of Reproductive Medicine, 45, 889–896.

31. Hillier S.L., Lipinsky C., Briselden A.M., Eschenbach D.A. (1993). Efficacy of intravaginal 0.75% metronidazole gel for the treatment of bacterial vaginosis. Obstetrics and Gynecology, 81, 963–967.

32. Hillier SL. (2005). The complexity of microbial diversity in bacterial vaginosis. New England Journal of Medicine, 353, 1886-1887.

33. Holmes K.K., Sparling P.F., Stamm W.E., Piot P., Wasserheit J.N., Corey L., Cohen S.M., Watts D.H. (2009). Sexually Transmitted Diseases. Yale Journal of Biology and Medicine, 82, 93.

34. Homayouni A., Bastani P., Ziyadi S., Mohammad-Alizadeh-Charandabi S., Ghalibaf M., Mortazavian A.M., Mehrabany E.V. (2014). Effects of Probiotics on the Recurrence of Bacterial Vaginosis: A Review. Journal of Lower Genital Tract Disease, 18, 79-86.

35. Homayouni Rad A., Vaghef E., Alipoor B., Vaghef L., Javadi M. (2012). Do probiotics act more efficiently in foods than in supplements? Nutrition, 28, 733-736.

36. Hyman R.W., Fukushima M., Diamond L., Kumm J., Giudice L.C., Davis R.W. (2005). Microbes on the human vaginal epithelium. Proceedings of the National Academy of Sciences, 102, 7952-7957.

37. Larsen B., Monif GR. (2001). Understanding the bacterial flora of the female genital tract. Clinical Infectious Diseases, 32, 69-77.

38. Lima M.F. Kierszenbaum F. (1985). Lactoferrin effects on phagocytic cell function increased uptake and killing of an intracellular parasite by murine macrophages and monocytes. Journal of immunology, 134, 4176-4183.

39. Livengood C.H., McGregor J.A., Soper D.E., Newton E., Thomason J.L. (1994). Bacterial vaginosis: efficacy and safety of intravaginal metronidazole treatment. American Journal of Obstetrics & Gynecology, 170, 759–764.

40. Livengood C.H., Soper D.E., Sheehan K.L., Fenner D.E., Martens M.G., Nelson A.L., Ismail M., Thorp J.M., Lappin M., Long B.J., Blackwelder T., Sweet R.L., Sagov S. (1999). Comparison of once-daily and twice-daily dosing of 0.75% metronidazole gel in the treatment of bacterial vaginosis. Sexually Transmitted Diseases, 26, 137–142.

41. Marcone V., Calzolari E., Bertini M. (2008). Effectiveness of vaginal administration of *Lactobacillus rhamnosus* following conventional metronidazole therapy: how to lower the rate of bacterial vaginosis recurrences. New Microbiologica, 31, 429-433.

42. Marcone V., Rocca G., Lichtner M., Calzolari E. (2010). Long-term vaginal administration of *Lactobacillus rhamnosus* as a complementary approach to management of bacterial vaginosis. International Journal of Gynecology & Obstetrics, 110, 223-226.

43. Marrazzo J.M., Koutsky L.A., Eschenbach D.A., Agnew K., Stine K., Hillier S.L. (2002). Characterization of vaginal flora and bacterial vaginosis in women who have sex with women. Journal of Infectious diseases, 185, 1307-1313.

44. Marrazzo J.M., Cook R.L., Wiesenfeld H.C., Murray P.J., Busse B., Krohn M., Hillier S.L. (2006). Women's satisfaction with an intravaginal Lactobacillus capsule for the treatment of bacterial vaginosis. Journal of Women's Health, 15, 1053–1060.

45. Martinez R.C., Franceschini S.A., Patta M.C., Quintana S.M., Candido R.C., Ferreira J.C., De Martinis E.C., Reid G. (2009). Improved treatment of vulvo-vaginal candidiasis with fluconazole plus probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14. Letters in Applied Microbiology, 48, 269–274.

46. Mastromarino P., Macchia S., Meggiorini L., Trinchieri V., Mosca L., Perluigi M., Midulla C. (2009). Effectiveness of Lactobacillus containing vaginal tablets in the treatment of symptomatic bacterial vaginosis. Clinical Microbiology and Infection, 15, 67-74.

47. Milani C., Hevia C., Foroni E., Duranti S., Turroni F., Lugli G.A., Sanchez B., Martı´n R., Gueimonde M., van Sinderen D., Margolles A., Ventura M. (2013). Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. Plos one, 8, 1-12.

48. Nugent R.P., Krohn M.A., Hillier S.L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. Journal of Clinical Microbiology, 29, 297–301.

49. Nyirjesy P., McIntosh M.J., Gattermeir D.J., Schumacher R.J., Steinmetz J.I., Joffrion J.L. (2006). The effects of intravaginal clindamycin and metronidazole therapy on

vaginal lactobacilli in patients with bacterial vaginosis. American Journal of Obstetrics Gynecology, 194, 1277–1282.

50. Ozkinay E., Terek M.C., Yayci M., Kaiser R., Grob P., Tuncay G. (2005). The effectiveness of live lactobacilli in combination with low dose oestriol (Gynoflor) to restore the vaginal flora after treatment of vaginal infections. British Journal of Obstetrics and Gynaecology, 112, 234-240.

51. Parent D., Bossens M., Bayot D., Kirkpatrick C., Graf F. (1996). Therapy of bacterial vaginosis using exogenously-applied Lactobacillus aciophili and a low dose of estriol: a placebo controlled multicentric clinical trial. Arzneimitell Forschung, 46, 68-73.

52. Pavlova S.I., Kilic A.O., Kilic S.S., So J.S., Nader-Macias M.E., Simoes J.A., Tao L. (2002). Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences. Journal of Applied Microbiology, 92, 451-459.

53. Pendharkar S., Magopane T., Larsson PG., Bruyn G., Gray GE., Hammarström L. (2013). Identification and characterisation of vaginal lactobacilli from South African women. Infectious Diseases, 26, 1,7.

54. Petricevic L., Unger F.M., Viernstein H, Kiss H. (2008). Randomized, double-blind, placebo-controlled study of oral lactobacilli to improve the vaginal flora of postmenopausal women. European Journal of Clinical Microbiology & Infectious Diseases, 141, 54-57.

55. Rampersaud R., Randis TM., Ratner AJ., (2012). Microbiota of the upper and lower genital tract. Seminars in Fetal & Neonatal Medicine, 17, 51-57.

56. Reid G., Bruce AW. (2001). Selection of Lactobacillus strains for urogenital probiotic applications. Journal of Infectious Diseases, 183, 77-80.

57. Reid G. (2001). Probiotic agents to protect the urogenital tract against infection. American Journal of Clinical Nutrition, 73, 437-443.

58. Reid G., Burton J., Hammond J.A., Bruce A.W. (2001). Nucleic acid based diagnosis of bacterial vaginosis and improved management using probiotic lactobacilli. Journal of Medicinal Food, 7, 223-228.

59. Reid G., Bruce A.W. (2003). Urogenital infections in women: can probiotics help? Postgrad Medicine Journal, 79, 428-432.

60. Rodríguez-Franco D.A., Vázquez-Moreno L., Ramos-Clamont Montfort G. (2005). Antimicrobial mechanisms and potential clinical application of lactoferrin. Revista Latinoamericana de Microbiologia, 47, 102-111.

61. Rönnqvist P.D., Forsgren-Brusk U.B., Grahn-Håkansson E.E. (2006). Lactobacilli in the female genital tract in relation to other genital microbes and vaginal pH. Acta Obstetricia Gynecologica Scandinavica, 85, 726-735.

62. Sanchez J., Campos P.E., Courtois B., Gutierrez L., Carrillo C., Alarcon J., Gutuzzo E., Hughes J., Watts D., Hillier S.L., Buchanan K., Holmes K.K. (2003). Prevention of sexually transmitted diseases (STDs) in female sex workers: prospective evaluation of condom promotion and trengthened STD services. Sexually Transmitted Diseases, 30, 273–279.

63. Schellenberg J., Links M.G., Hill J.E., Dumonceaux T.J., Peters G.A., Tyler S., Ball T.B., Severini A., Plummer F.A. (2009). Pyrosequencing of the chaperonin-60 universal target as a tool for determining microbial community composition. Applied Environmental Microbiology, 75, 2889-2898.

64. Sehgal R., Goyal K., Sehgal A. (2012). Trichomoniasis and Lactoferrin: Future Prospects. Infectious Diseases in Obstetrics and Gynecology, 1-8, Article ID 536037.

65. Shalev E., Battino S., Weiner E., Colodner R., Keness Y. (1996). Ingestion of yogurt containing *Lactobacillus acidophilus* compared with pasteurized yogurt as prophylaxis for recurrent candidal vaginitis and bacterial vaginosis. Archives of Family Medicine, 5, 593-596.

66. Shau H., Kim A., Golub S.H. (1992). Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. Journal of Leukocyte Biology, 51, 343-349.

67. Shi Y., Chen L., Tong J., Xu C., (2009). Preliminary characterization of vaginal microbiota in healthy Chinese women using cultivation-independent methods. Journal of Obstetrics and Gynaecology Research, 35, 525-532.

68. Sobel J.D., Ferris D., Schwebke J., Nyirjesy P., Wiesenfeld H.C., Peipert J., Soper D., Ohmit S.E., Hillier S.L. (2006). Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. American Journal of Obstetrics and Gynecology, 194, 1283–1289.

69. Stahl C.E., Hill G.B. (2009). The clinical perspectives in obstetrics and gynecology. Galask RP, editor. Infectious Diseases in the Female Patient. pp. 16–42.

70. Tarek M.M., Alaa El-Din E.E., Gehan K. E., Mai Salah E.K. (2014). Study on the Therapeutic Benefit on Lactoferrin in Patients with Colorectal Cancer Receiving Chemotherapy. International Scholarly Research Notices, 1-10, Article ID 184278.

71. Tärnberg M., Jakobsson T., Jonasson J., Forsum U. (2002). Identification of randomly selected colonies of lactobacilli from normal vaginal fluid by pyrosequencing of the

16S rDNA variable V1 and V3 regions. Acta Phatologica Microbiologica e Immunologica Scandinavica, 110, 802-810.

72. Thies F.L., König W., König B. (2007). Rapid characterization of the normal and disturbed vaginal microbiota by application of 16S rRNA gene terminal RFLP fingerprinting. Journal of Medical Microbiology, 56, 755-761.

73. Venkatesh, M.P., Rong, L. (2008). Human recombinant lactoferrin acts synergistically with antimicrobials commonly used in neonatal practice against coagulase-negative *staphylococci* and *Candida albicans* causing neonatal sepsis. Journal of Medical Microbiology, 57, 1113-1121.

74. Verhelst R., Verstraelen H., Claeys G., Verschraegen G., Delanghe J., Van Simaey L., De Ganck C., Temmerman M., Vaneechoutte M. (2004). Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis. BMC Microbiology, 4, 16-27.

75. Vitali B., Pugliese C., Biagi E., Candela M., Turroni S., Bellen G., Donders GG., Brigidi P. (2007). Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidiasis, or no infection, analyzed by PCR-denaturing gradient gel electrophoresis and real-time PCR. Applied of Environmental Microbiology, 73, 5731-5741.

76. Witkin S.S., Linhares I.M., Giraldo P. (2007). Bacterial flora of the female genital tract: function and immune regulation. Best Practice & Research Clinical Obstetrics & Gynaecology, 21, 347-354.

77. Ya W., Reifer C., Miller L.E. (2010). Efficacy of vaginal probiotic capsules for recurrent bacterial vaginosis: a double-blind, randomized, placebo-controlled study. American Journal of Obstetrics and Gynecology, 203, 1–6.

78. Yamamoto T., Zhou X., Williams CJ., Hochwalt A., Forney LJ. (2009). Bacterial populations in the vaginas of healthy adolescent women. Journal of Pediatricand Adolescent Gynecology, 22, 11-18.

79. Zhou X., Bent S.J., Schneider M.G., Davis C.C., Islam M.R., Forney L.J. (2004). Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology, 150, 2565-2573.

80. Zhou X., Brown C.J., Abdo Z., Davis C.C., Hansmann M.A., Joyce P., Foster J.A., Forney L.J. (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J* 1, 121-133.

Chapter 5

Lactobacillus rhamnosus GG supplementation in Systemic Nickel allergy Syndrome patients

Alessandra Pino, Cinzia Lucia Randazzo, Cinzia Caggia, Erwin Zoetendal, Willem de Vos.

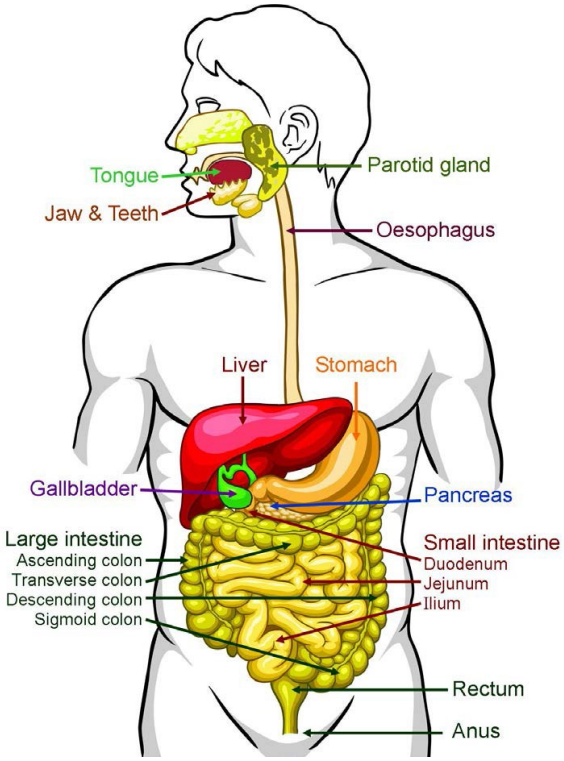
Manuscript for publication in preparation

GENERAL OVERVIEW

THE GASTRO-INTESTINAL TRACT MICROBIOTA

The human gastro-intestinal tract (GIT) is divided into the oral cavity, esophagus, stomach, small intestine, colon, rectum and anus. The small intestine is composed of the duodenum, jejunum and ileum. The GIT together with the associated organs, salivary glands, liver, gallbladder and pancreas constitute the digestive system (Figure 5.1). The ladder is responsible for the breakdown and modification of food into smaller portions and usable nutrients, electrolytes, and fluids. In addition, it excretes unabsorbed residues, provides a protective barrier against the entry of toxic substances and infectious agents, serves as the largest endocrine organ in the body and interacts with other endocrine organs: the nervous system, the circulatory system, the immune system, and so on (75).

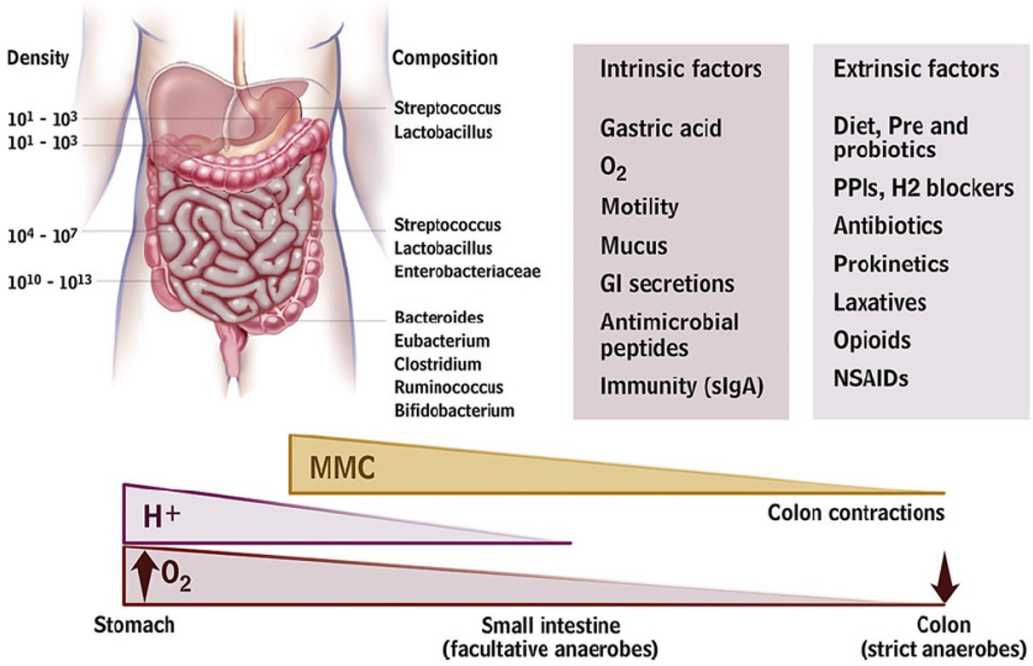
Figure 5.1 Schematic representation of the human digestive system.



The human GIT is colonized by Archaea, Eukarya and a dense and complex community of bacteria all of which have a large impact on the health of the host through: the modulation of the immune system, the protection against pathogens, the development of the intestinal microvilli, the enteric nerve regulation, the promotion angiogenesis and the conversion of nutrients and metabolites (77). The total number of microorganisms in the GIT is around 10^{14} and varies greatly between different regions. The intestinal microbiota differs quantitatively and qualitatively, increasing in number and population diversity along the length of the GIT. Moreover, there are also significant differences between the microbiota present in the gut lumen and the microbiota attached to and embedded in the mucus layer of the GI tract (66). The vast majority of the intestinal bacteria reside in the colon, reaching values between 10^{12} to 10^{13} bacteria per gram of faeces (figure 5.2). The composition of the different sites of the intestinal tract varies due to different environmental factors, such as pH, transit time, mucus layer and peristaltic movements. In fact, the bacterial load increases and the oxygen levels declines towards the distal part of the digestive tract, while the pH rises from acidic towards neutrality. Concerning the composition, the GI microbiota is taxonomically classified via the traditional biological nomenclature (Phylum-Class-Order-Family-Genus-Species) and currently more than 50 bacterial phyla have been described, of which 10 inhabit the colon and three predominate: the *Firmicutes*, *Bacteroidetes* and the *Actinobacteria* (15, 84). Although the GI tract microbiota predominantly contains bacteria (94%), there are also viruses (4.8%) and archaea (0.5%) (3). In detail, the bacterial population, though demonstrating great variation from subject to subject, contains over 90% of *Firmicutes* and *Bacteroidetes*. Additionally *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* are among the most abundant phyla (50, 38,). Healthy subjects are reported to harbour around 400-600 different species in their colon

(51, 68, 50, 61). In any case, the definition of health and healthy intestine does not take into account the vast natural variation and subject-specificity of the intestinal microbiota. Host genetic and immune as well as environmental factors influence intestinal microbiota composition, which in turn shape host immunity and physiology within and beyond the GIT (figure 5.2). It has been shown that the relative abundance of both *Firmicutes* and *Bacteroidetes* can vary between 0 to 99% (31) and the most abundant phylotypes can differ by as much as 5000-fold between subjects (30). A number of host mechanisms participate in the GIT microbiota modulation, including gastric acid secretion, fluid, GI motility, anticommensal sIgA and antimicrobial peptide production. Drugs that block acid secretion and affect GI motility can indirectly alter the microbiota. Antibiotics, depending on spectrum and dosage, as well as dietary modifications, including probiotic and fibre supplements, will directly affect microbiota composition.

Figure 5.2 Gut microbiota and the intrinsic end extrinsic factors that can affect its distribution and composition.

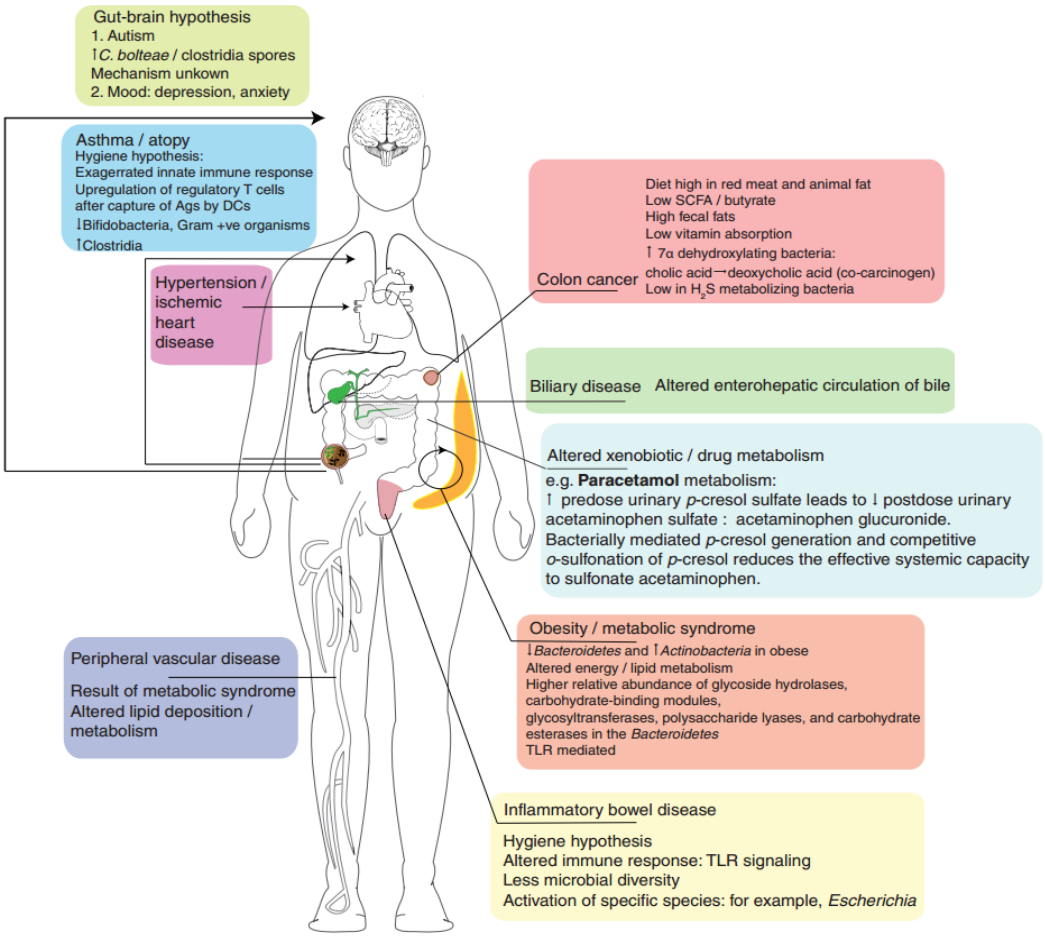


MMC, migration motor complexes; H^+ hydrogen ions; O_2 partial oxygen tension; slgA secretory immunoglobulin; PPI proton pump inhibitor; NSAID non-steroidal anti-inflammatory drug.

Each individual's microbiota is highly specific (83, 13, 73, 70). This high subject-specificity has been shown to be a combination of environmental factors, shaping the microbiota immediately after birth, as well due to genetic variation (5, 32, 33). From the ecological point of view, resilience represents a community's ability to respond to perturbations by resisting changes and the ability to recover to its original form (28). The capability for the microbiota of healthy subjects to maintain its composition in time has been associated to health in several studies. It has been shown that the microbiota retains its similarity over time, both the short and long term (83, 76, 14, 44, 62, 67, 35, 82, 43). It is also known that there is little flux in the

presence of the species but their abundances vary in time (54) and the temporal variation is associated to subjects' microbial diversity (23). The majority of the current data support the view that the intestinal microbiota of healthy adults operates in a state of homeostasis and shows resilience to perturbations such as lifestyle changes, medication and diet. Moreover, the gut microbiome has been directly implicated in the etiopathogenesis of a number of diverse pathological states as obesity (72), circulatory disease (29), inflammatory bowel diseases (IBDs) (42) and autism (22) (Figure 5.3).

Figure 5.3 Diseases influenced by GIT microbiota.



Kinross 2011 (34)

APPROACHES TO STUDY INTESTINAL MICROBIOTA

Most of the bacterial species colonizing the human intestine are strictly or facultative anaerobic and have physiologically adjusted to the conditions residing in the GIT. These conditions are difficult to mimic under laboratory conditions. In spite of the fact that over than 1000 species have been cultured from the human intestinal tract (55), an important fraction (estimated to be approximately 70%) of the intestinal microbes have not been cultured or identified yet (24). Therefore, the true diversity of intestinal microbiota has remained incompletely covered by using traditional cultivation strategies or molecular methods with low resolution, whereas the rapid development of culture-independent high-throughput molecular applications, during the recent years, has enabled the deep and comprehensive analyses of total microbiota diversity. These high-throughput techniques are based on either direct sequencing of nucleic acids in the sample or on their detection by using high-density oligonucleotide arrays. Phylogenetic microarray is a high-throughput platform designed for the simultaneous detection of thousands of DNA or RNA sequences. Further, it allows the concurrent analysis of numerous samples, making microarrays a fast, cheap and user-friendly technology. As other molecular methods for microbiota community analysis, this technology is most often based on sequences of variable regions of the 16S rRNA gene, denoting that the sequence knowledge is a prerequisite for the array design. Phylogenetic microarrays have been shown to have higher sensitivity than high-throughput sequencing strategies (12, 71) and to detect bacterial DNA as low as 0.00025 % of the sample (48). The major challenge in this technology is the possibility of cross-hybridizations, i.e. that probes hybridize also to highly similar non-target sequences (24). Several phylogenetic microarrays targeting the human intestinal microbiota have been developed (48, 52, 9, 71) and the Human Intestinal

Tract Chip (HITChip) being one of the most intensively used. The HITChip enables rapid and sensitive profiling of the microbiota diversity and allow the relative quantification of all bacterial groups at different taxonomic levels simultaneously (53). The so-called next-generation sequencing methods deliver sequences from 16S rRNA gene amplicons or from total community DNA. Direct sequencing of partial 16S rRNA gene amplicons (massively parallel sequencing) is able to sequence massive amount of sequences at the same time in the same reaction. Due to the sequencing of short reads (typically around 100 bp), it is possible to increase the amount of sequences analysed, thus enabling also the detection of low abundant bacteria (24). This method gives phylogenetic and quantitative information and enables detection of unknown bacteria. Massively parallel sequencing is fast but requires intense computational data analysis that includes the removal of chimeric sequences (24). One overlooked issue is the generation of errors as is illustrated by the need for developing a low error 16S rRNA amplicon sequencing method to be able to monitor the microbiota of subjects over time (difficulties in identifying subjects based on their faecal microbiota (20), while this was done easily with the HITChip (54). While advances in molecular microbiological techniques allow a high-throughput analysis of the microbiota, several problems are related to inappropriate use of these 16S rRNA gene sequence -based technologies. Sufficient pre-treatment of faecal samples, such as sample collection procedures and storage conditions, efficient nucleic acid extraction and unbiased PCR amplification are crucial in order to cover the full microbial diversity (45). In addition, identification of sequences based on very short reads is not always reliable (80). Despite the powerful molecular techniques for microbiota characterization, culturing is still utmost valuable as it is a necessary step for the detailed physiological and biochemical identification of individual isolates. Experimental

characterization of isolates and their metabolic activities are needed to expand the reference databases used for the annotation of metagenomic sequence data, because currently a significant proportion of obtained data is lacking a close match in the reference databases (36, 79). In future, high-throughput cultivation of new species in combination with metagenomics will give us new insights into microbiota composition and function (55).

Intestinal microbiota composition in Systemic Nickel Allergy Syndrome (SNAS) patients: a high throughput microarray analysis.

INTRODUCTION

Nickel (Ni) is a ubiquitous highly sensitizing metal, which can trigger allergic manifestations. Several evidences prove that Ni, in experimental animal models, can induce negative effects on the immune system, but the data on immunomodulation are contradictory: among the effects most frequently encountered prevail the suppression of the immune response. The exposure to Ni can occur not only through the contact with the skin but also by the gastrointestinal absorption. In fact, many foods have a high content of Ni. Vegetables represent the main dietary source. Ni is mainly concentrated in the reproductive part of plants (hazelnuts, peanuts, wheat grains, legumes) and in a normal diet 50% of the daily requirement of Ni is provided by cereals and legumes, followed by fats, dairy products and fruit. The average daily intake of Ni is approximately 400-600 µg. It is estimated that about 10-20% of the worldwide population are allergic to Ni (69). Nickel allergy can present itself in various forms: cutaneous, localized or systemic, and extra-cutaneous. Schematically we can distinguish between the Allergic Contact Dermatitis (ACD) and the Systemic Nickel Allergy Syndrome (SNAS) that can have cutaneous and/or extra cutaneous signs and symptoms (gastrointestinal, respiratory, neurological, etc.). SNAS is an emergent chronic inflammatory disorder that has been defined as a definite condition only recently (25, 46, 8, 11, 17, 57). Twenty percent of the ACD patients also experience urticaria and angioedema, flares, itching, cough, headache and gastrointestinal symptoms due to the ingestion of Nickel-rich foods (16, 25, 46). Gastrointestinal symptoms and cutaneous manifestations in areas not in contact with

Ni, recurrent infections and an increase in oxidative stress are the principal SNAS manifestations. Women are strongly affected then man with a high ratio (female/male) ranging between 3:1. Christensen (10) was the first author to suspect that ingested Ni could be responsible for these reactions. The diagnosis of SNAS, both cutaneous (systemic contact dermatitis) and extra-cutaneous is far more complex than the diagnosis of ACD. The only possibility of definitive diagnosis is one that mimics natural exposure, i.e. the exposure or provocation test. In the case of exposure via food, the diagnostic procedure would consist of, as for all allergies and food intolerances, an oral provocation test with the suspected food after the diagnostic elimination diet of the same food. Any other tests, such as the patch as well as the prick with Nickel sulphate, sometime performed, are only indicative but are not considered diagnostic (49). In 2009, Rosato and co-workers have demonstrated that Nickel-hypersensitivity is associated with a significant increase in susceptibility to viral, bacterial and fungal infections (58). In fact, in Nickel-sensitive patients a higher incidence of recurrent herpes labialis, urinary tract infections, genital candidiasis and upper respiratory tract infections was detected. Moreover, the same authors have found a reduction in the number of annual episodes of herpes labialis in patients following Nickel-poor diet (58). A successive study conducted by Rosato and co-workers in 2011 on pediatric patients, showed higher incidence of recurrent upper respiratory tract infections and recurrent otitis media in children with Ni-ACD (59). This susceptibility, according to the authors, could be determined by a reduction of the natural killer (NK) cell activity caused by the immunomodulatory effects of Ni in allergic subjects. Regarding the headache and the gastrointestinal symptoms, the data of literature are conflicting and not efficiently supported. However the gastrointestinal symptoms data seem to be more accurate than headache ones (49). An increase in oxidative

stress in Ni-sensitive patients was demonstrated by Gangemi (2009) and co-workers. Serum concentrations of protein carbonyl groups (PCGs) and nitrosylated proteins (NPs), biomarkers of oxidative stress, were measured before and after oral Nickel challenge in thirty-one women with diagnosis of Ni-ACD and in thirty-one healthy controls. Although both Nickel-allergic patients and controls presented similar serum levels of PCGs, NP values in Nickel-sensitive patients appeared higher than in controls and tended to decrease after the challenge (25). Finally, pivotal clinical studies, monocentric non-randomized and non-double-blind controlled, have preliminarily shown that the oral hyposensitization procedure, obtained with the administration of increasing doses of Ni sulfate, could be suitable to induce immune-tolerance and symptoms improvement (46, 11). However, further large randomized clinical trials are needed to confirm these preliminary and suggestive clinical data and to evaluate the effects of hyposensitization therapy. Andrioli and co-workers (2) documented a twofold prevalence of Chronic autoimmune thyroiditis (CAT) in SNAS subjects compared to that observed in patients with no-SNAS immune diseases (26.5 vs. 12.7 %, $p<0.01$). This result is remarkable but remains unclear due to the different immunopatogenetic mechanisms underlying the two diseases. Past studies suggested that environmental factors, such as high iodine intake, selenium deficiency, drugs and pollutants may be implicated in the development of CAT (18). Moreover, metals had been reported to be implicated in the immune process and in inflammation (18). Therefore, Nickel plays a crucial role in SNAS and it may be potentially involved in the development of CAT, perhaps by increasing thyroid antigenicity and promoting the progression of the autoimmune response in susceptible individuals. Focused studies are mandatory to support these speculative hypotheses and a possible shared genetic background. In fact, CAT is supposed to be related to variants in the

human leucocyte antigen (HLA) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) genes (21), whereas data on the genetic predisposition of SNAS are scanty, being the association with DR7-DQ2.2 haplotype the most frequent finding (11). To date, poor are the information concerning the possible imbalance of intestinal microbiota in SNAS patients and only one study (56) is now available. In 2014 our research group conducted a preliminary double blind randomized placebo-controlled study in order to evaluate the effect of probiotic *Lactobacillus reuteri* DSM 17938 strain supplementation in terms of modulation faecal LAB population linked to a reduction of gastro intestinal and cutaneous symptoms and to an increase of patient's quality of life. The approach applied allowed us to identify the dominant LAB species in patients suffering from SNAS and to assert that the *L. reuteri* DSM 17938 strain is able to influence significantly both the LAB dynamics and the clinical symptoms (56).

As previously reported, the literature in relation to SNAS is very poor and only a few clinical studies were conducted. Up to now, no information are available concerning the intestinal microbiota composition and its relation with gastro-intestinal symptoms. The present study investigated for the first time the microbiota composition of SNAS patients. The advanced throughput HITChip microarray was applied in order to: characterize the intestinal microbiota composition of SNAS patients, assess the possible existence of a potential syndrome specific microbiota, evaluate the changes occurred after low Nickel diet and desensitization treatment alone or in combination with *Lactobacillus rhamnosusu* GG supplementation.

MATERIAL AND METHODS

Patients enrolment

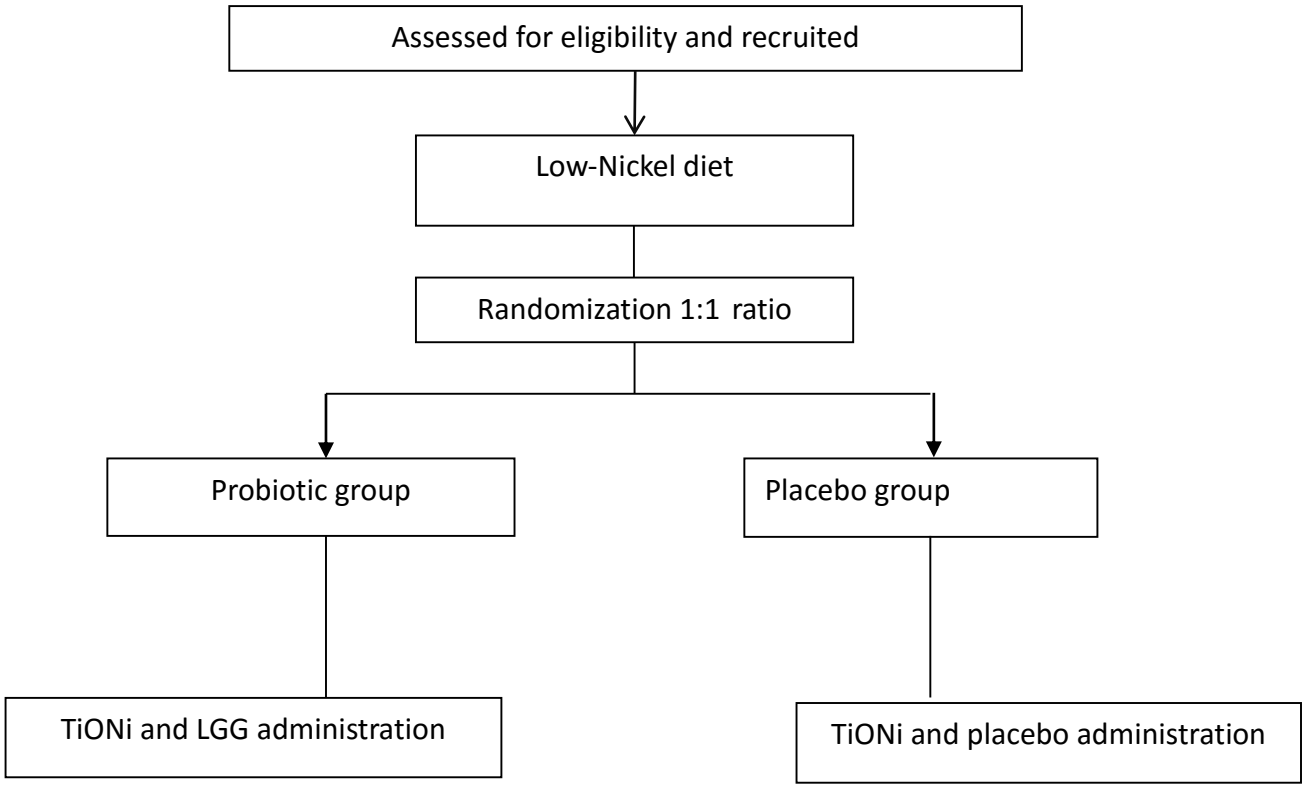
The present study was a multicentre randomized controlled trial with two parallel groups: probiotic and placebo. The patients were recruited using the following criteria: age, positivity to a Ni-patch and prick-test, presence of GI reactions, such as symptoms related to meals as dyspepsia, nausea and/or vomiting, type of stools (frequency and consistency), recurrent abdominal pain (RAP) and cutaneous reactions such as urticaria, itching and eczema after ingestion of Ni containing food. Exclusion criteria included organic diseases (as established by medical history, complete blood count, urinalysis, stool examination for occult blood, ova and parasites, blood chemistries, abdominal ultrasound, breath hydrogen testing and endoscopy if needed), presence of other chronic disease such as celiac disease, pregnancy and lactation, concomitant treatment with steroids and/or antihistamines, participation in another study. Patients were not allowed to use gastric acid inhibitors, laxatives, anti-diarrhoeal medications or antibiotics for at least 1 month before the beginning of the study and systemic or topic antihistamines or corticosteroids during the study. Moreover, the consumption of probiotics or prebiotics for at least 1 month before the study were not allowed.

Study design

Sixty patients satisfied the inclusion criteria previously reported, were recruited and subjected to a Low-Ni diet for 1 month then, patients that presented the improvement of symptoms at least 70% from baseline were randomized in two groups: probiotic group (P) or placebo group (C) (figure 5.4). Both groups received, after Low Ni-diet, Ni as $NiSO_4 \cdot 6H_2O$ with microcrystalline cellulose as excipient (NiTOH) for 3 months. During the NiTOH administration, probiotic

groups received 1 daily tablet (10^9 cfu ml^{-1}) of *Lactobacillus rhamnosus GG,* ATCC 53103 probiotic strain, and the placebo group received 1 daily placebo tablet, all identical in appearance and flavour, both gently provided by DICOFARM SpA (Roma, Italy). The study was divided in four times of intervention: baseline (T1), after 1 month of Low Ni-diet (T2), 3 months probiotic or placebo feeding (T3) and 2 months after the end of the treatment (T4). The following tertiary hospitals participated to the trial. Allergy and immune tossicology unit, G. d'Annunzio University Foundation, Chieti, Italy; Allergology and Clinical Immunology, G. Martino Hospital, University of Messina; Catholic University of the Sacred Heart Institute of Internal and Geriatric Medicine Milano, Italy; Respiratory Allergology Unit Respiratory disease Institute, University of Catania, Italy; Merate Hospital, Lecco, Italy. The study was conducted in accordance with the Helsinki Declaration (2000). The trial was approved by the local Ethics Committee and informed written consent was obtained from all participants prior to enrolment.

Figure 5.4 Study design



Faecal samples collection

Faecal samples were collected, in duplicate, at T1, T2, T3 and T4 sampling times using sterile plastic containers. Collection was performed every Thursday (± 1 day), to avoid major influences of changing dietary habits during the weekend. Stool samples were frozen immediately after collection and stored in -80 °C until further processing.

DNA extraction

Faecal samples were melted on ice and 0.25 g of faeces was weighted into a 2 ml screw-cap tube and mixed with 1 ml of lysis buffer, four glass-beads (3.0 mm, Lenz Laborglas GmbH & Co., Germany) and 0.5 g of zirconia beads (0.1 mm, Biospec Products, Inc., USA) then homogenized with two rounds of bead-beating with FastPrep-24 (FP120-230, Bio 101 ThermoSavant, Holbrook, NY). Treatment time was 3 min in both bead beating rounds and samples were putted on ice between. Lysate fraction obtained from first homogenization step was removed before performing the second bead-beating round. The lysis buffer used in the RBB-steps was 4% (w/v) sodium dodecyl sulphate (SDS), 50 mM Tris-HCl (pH 8), 500 mM NaCl and 50 mM EDTA. After RBB-step, DNA precipitation was done adding ammonium acetate and isopropanol. Nucleic acids were washed with 70% Ethanol, pelleted and subsequently dissolved in TE buffer. DNA was further purified by RNase A and proteinase K treatments and by using the purification columns and buffers from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA was purified using the One step PCR inhibitor Removal Kit (Zymo). DNA yield was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE). DNA concentration was adjusted to 20 ng ml^{-1} and was used as template for PCR amplification.

PCR amplification

The total bacterial 16S rRNA genes were amplified from genomic DNA by using the FastStart Taq DNA polymerase, dNTPack PCR amplification kit and the primers *T7prom*-Bact-27-for (5'-TGA ATT GTA ATA C GA CTC ACT ATAGGG GTT TGA TCC TGG CTC AG-3') and Uni-1492-rev (5'-

CGG CTA CCT TGT TAC GAC-3'), which ensured the introduction of a T7 promoter sequence at the 5' terminus of the rRNA gene amplicon. PCR reactions were carried out in a final volume of 50 µl, and 10 ng of DNA samples was used as template. Samples were initially denatured at 94°C for 2 min followed by 35 cycles of 94°C (30 s), 52°C (40 s), 72°C (90 s) and a final extension at 72°C for 7 min. The PCR products were purified using the High pure PCR Product Purification Kit (Roche) according to the manufacturer's instruction. PCR success and amplicons size were verified running the products on a 1% agarose gel, 20 min at 100V (Lonza). Final DNA concentration was determined using a NanoDrop spectrophotometer as described above.

RNA production

In vitro transcription of DNA into RNA was performed using RNAMaxx T7 Transcription Kit (Ambion, Austin, Tx, USA). The reaction mix was performed as follow: 5X RNAMaxx trascriptomic buffer; rATP, rCTP, rGTP, rUTP each 100mM; dithiothreitol 0.75M; yeast inorganic pyrophosphatase (0.75 U/µl); T7 RNA polymerase (200 U/µl); ammino-allyl rUTP; RNase block; 500 ng of the T7-16S rRNA gene PCR-product. The transcription reaction was performed at 37°C for 60-90 min, then the 1 µl of DNAse was added and the reaction was again incubated at 37°C for 15 min. The RNA was purified using the RNeasy Mini-elute clean-up kit (Qiagen, Hilden, Germany) according to the following protocol. The reaction was equilibrated with nuclease free water and mixed with 350 µl RLT buffer then vortexed and briefly centrifuged. 250 µl of 100% Ethanol was added and mixed by pipetting then the solution was transferred to the column (provided in the kit) and centrifuged for 10s at 8,000 xg (RCF value). 500 µl of RPE buffer was added and the solution was again centrifuged for 10s at 8,000 xg. After replacing the collection tube 500 µl of 80% ethanol was added and

centrifuged for 2 min at 8,000 xg. A new centrifugation was performed for 5 min at 13,000 xg with column caps opened then 15 µl of nuclease free water was directly added on the filter and leave for 1 min on bench. The RNA was obtained after centrifugation for 1 min at 8,000 xg. RNA yield was measured as described above then stored at -80^{o}C until further use.

Fluorescent labelling

Amino-allyl-modified nucleotides were coupled with CyDye using the Post-Labelling Reactive Dye (Amersham Biosciences, Little Chalfont, UK), previously dissolved in 84 µl dimethyl sulfoxide. Labelling reactions were performed in a 25 mM sodium bicarbonate buffer (pH 8.7) by adding 20 µl of dissolved CyDye to 2 µg of purified RNA in a final volume of 40 µl. Samples were incubated for 90 min in the dark at room temperature. The reaction was stopped by adding 15 µl of 4 M hydroxyl-amine and incubating for 15 min in the dark. Labelled RNA was purified and quantified as described above.

Hybridization

100 ng of each Cy3 and Cy5 samples were mixed with 5 µl 10x Blocking Agent (Agilent) and nuclease free water to a volume of 24µl. RNA was fragmented by adding 1 µl of 25x fragmentation buffer (Agilent) and the mixture was incubated for 30 min at 60 ^{o}C. After cooling on ice, 25 µl of freshly prepared hybridisation buffer was added. The hybridization buffer contained 20X SCC Buffer (GIBCO), ultrapure 10% SDS (Invitrogen), Triton X-100 (Sigma), distilled water (GIBCO). 40 µl of the prepared hybridization mix were added to the center of

Agilent slide cover and the latter was joined with the array. Covered array was placed in the hybridization chamber and incubated in the hybridization oven preheated at 62.5^{o}C overnight.

Scanning

Slides were subjected to three washing steps each for 10 min at room temperature. Each step was performed using the following washing solution: 0.1x SSC, 1x SSC and 0.06x SSPE. The slides were shortly dipped in acetonitril and carefully removed then scanned in Agilent Microarray Scanner.

Microarray analysis

Data were extracted from microarray images and intensity values for each spot were quantified by using Agilent Feature Extraction software, version 9.5 (www.agilent.com). This software determines the positions of each spot on the Agilent microarray based on auto-grid and auto-spot finding algorithms, performs local background calculation and subtracts it from the hybridization signal of each spot. The array normalization was conducted (53, 60) including spatial normalization with polynomial regression of each scanner channel followed by outlier detection and quantile normalization of both of the Cy3 and Cy5 dyes for each sample. The HITChip signal intensity was analysed using the following pylogenetic assignment levels: 1) the phylum-level, with the specification of Firmicutes down to Clostridium cluster, creating altogether 23 groups; 2) the genus-like level, including 131 groups of sequences with ≥ 90% sequence identity, and 3) the phylotype (species-like) level with 1033 distinct phylotypes with ≥ 98% sequence similarity to cultured species or clones corresponding to uncultured

microorganisms. Genus-level taxa with ≥ 90% sequence identity distributed over multiple genera are termed "*et rel*."

Data analysis

The HITChip data analysis was performed in R version 3.2.2, Canoco 5, and Microsoft Excel. The bacterial composition of the samples was compared at phylum level and at the approximate genus level using the Wilcoxon signed-rank test adjusted by Benjamini-Hochberg (BH) for false discovery rate (FDR) correction, in which a corrected $P<0.05$ was considered significant. The diversity of the microbial profiles was assessed by computing the Simpson's reciprocal index of diversity (63) and the Shannon index of diversity (27). Morever, richness and evenness were also evaluated. Hierarchical clustering, computed with Pearson correlation distance metric, and heat map analysis were used to group the samples according to the correlation distance and average clustering criteria. Multivariate statistical analysis, Principal Component Analysis (PCA) and Redundancy Analysis (RDA) were performed with Canoco 5.

RESULTS

Compositional microbiota analysis at baseline

A total of 55 subjects were investigated at baseline. The population consisted of 51 female and 4 male, the age ranged from 17 to 63 years old (supplementary information). HITChip analysis detected 1025 species-like groups, 130 genus-like groups and 22 phylum-like groups in the faecal samples of the studied population. The relative contribution of the major phyla in the faecal samples of patients at baseline (T1 sampling time) are plotted in figure 5.5. The

most abundant phylum consisted of *Firmicutes* with 85% contribution to the total microbiota, followed by an average 7% *Actinobacteria*, 5% *Bacteroides* and 1% *Proteobacteria*. *Asteroleplasma*, *Cyanobacteria*, *Fusobacteria*, *Spirochaetes* and *Verrucomicrobia* were detected with proportional share below 0.5% (data not shown). As reported in figure 5.6, whereas the aggregate microbiota was composed as previously reported, the individual composition datasets showed strange variation for all phylum. At genus-like level thirty-four genera showed relative contribution greater that 0.5%. As expected, the majority of the genera belonged to Clostridium cluster XIVa and Clostridium cluster IV. In detail, as showed in figure 5.7 the genera, belonging to *Clostridium* cluster XIVa, that showed the higher occurrence were *Ruminocossus obeum* et rel. and *Coprococcus eutactus* et rel., followed by *Anaerostipes caccae* et rel., *Dorea formicigenerans* et rel., and *Eubacterium hallii* et rel. Among the *Clostridium* cluster IV the dominant genus was *Subdoligranulum variabile* et rel. followed by *Clostridium cellulosi* et rel., *Oscillospira guillermondii* et rel., *Prevotella melaninogenica* et rel., *Clostridium leptum* et rel., *Clostridium orbiscindens* et rel., *Ruminococcus bromii* et rel., and *Sporobacter termitidis* et rel. Variation of individual composition at genus-like level is reported in figure 5.8. At species-like level 191 of the total 1025 species detected, showed relative contribution greater that 0.1% as reported in figure 5.9. The species presented the major occurrence were in particular *Subdoligranulum variabile* and *Ruminococcus bromii* followed by species belonging to the *Streptococcus* genus (*S. thermophilus, S. salivarius, S. sanguis and S. pneumoniae*). *Butyrivibrio fibrisolvens*, *Dorea longicatena*, *Eubacterium rectale*, *Eubacterium hallii*, *Clostridium symbiosum* and *Eubacterium ventriosum* were found as the main species belonged to the Clostridiun cluster XIVa. *Bacteroides fragilis*, *Bacteroides vulgatus* and *Bacteroides uniformis* were the main exponents of the Bacteroidetes phylum.

Several species, such us *Bifidobacterium angulatum*, *B. longum*, *B. infantis*, *B. adolescentis, Collinsella aerofaciens* and *B. pseudocatenulatum*, belonged to the Actinobacteria phylum.

Figure 5.5 Relative contribution of phylum like level to the faecal microbiota of SNAS patients at baseline.

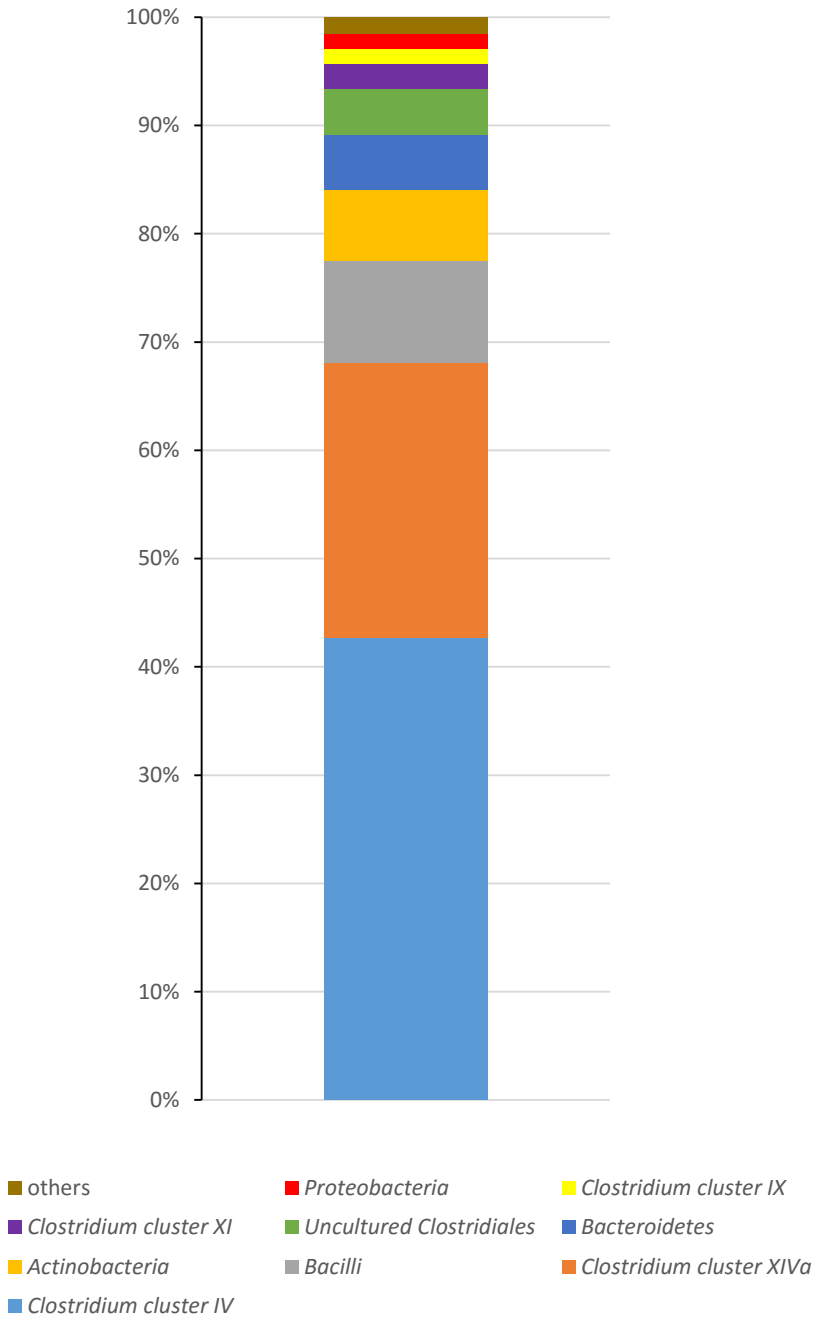


Figure 5.6 Individual microbiota composition at phylum-like level of SNAS patients at baseline.

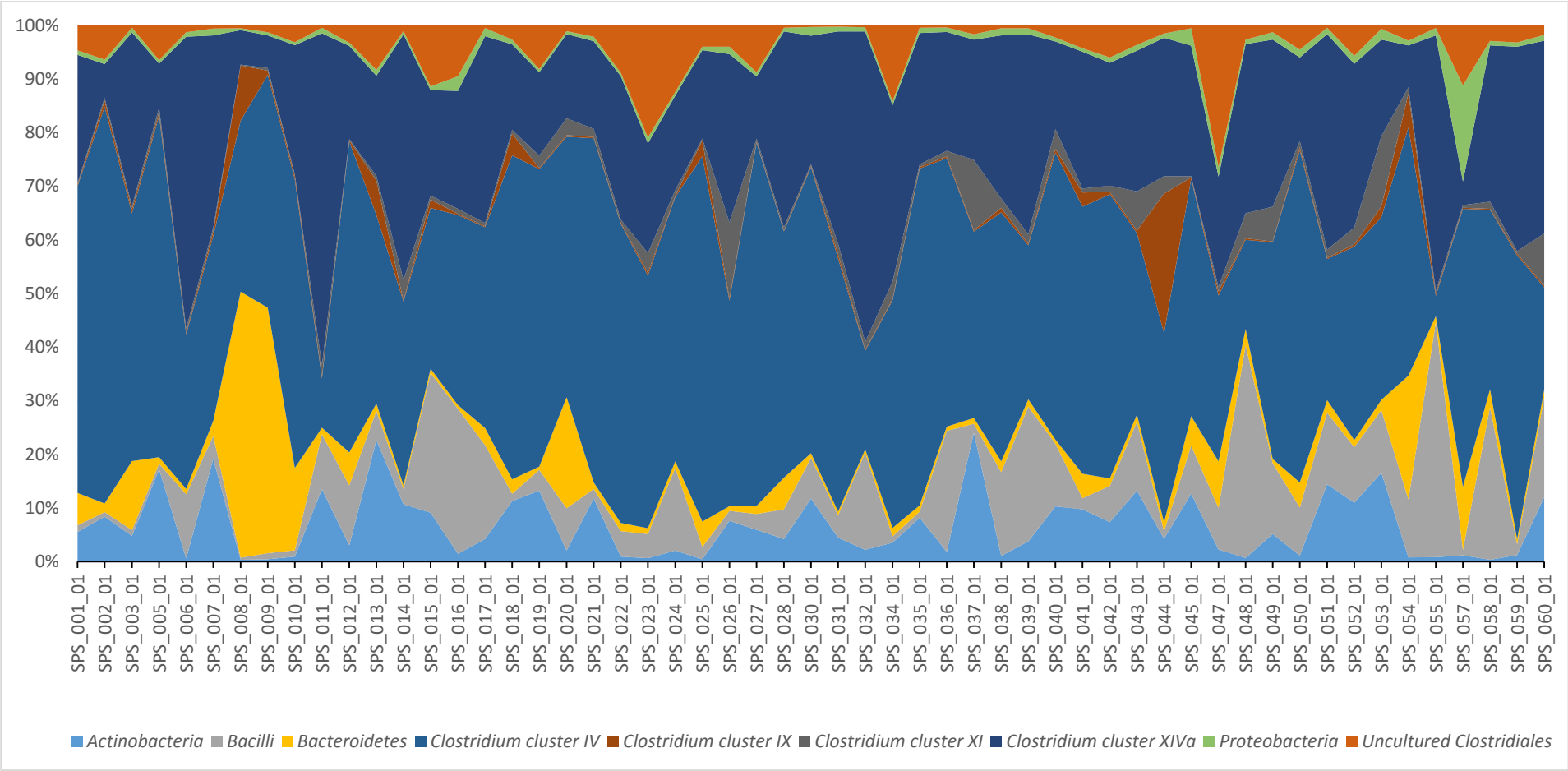


Figure 5.7 Relative contribution of genus like level to the faecal microbiota of SNAS patients at baseline.

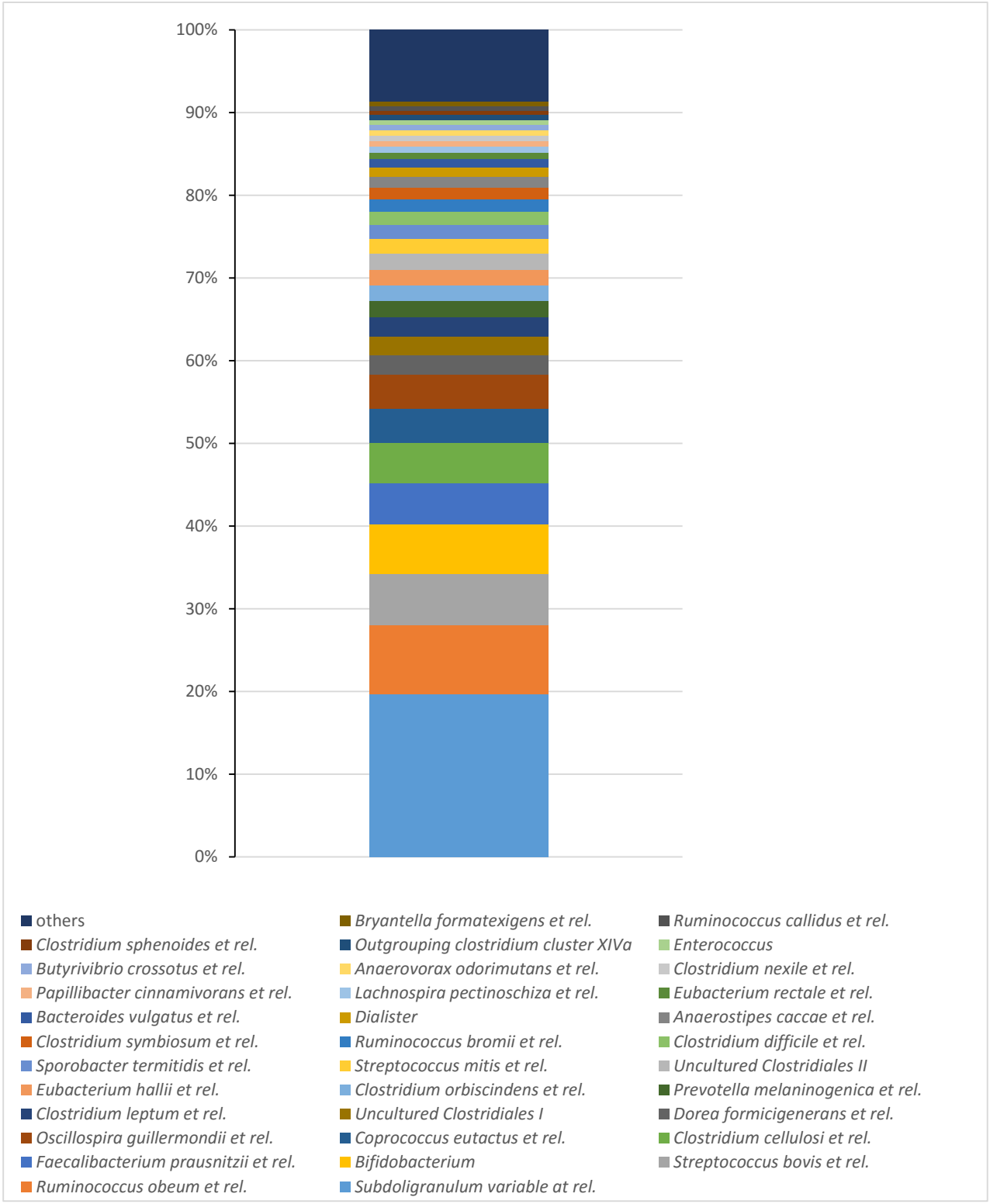


Figure 5.8 Individual microbiota composition at genus-like level of SNAS patients at baseline.

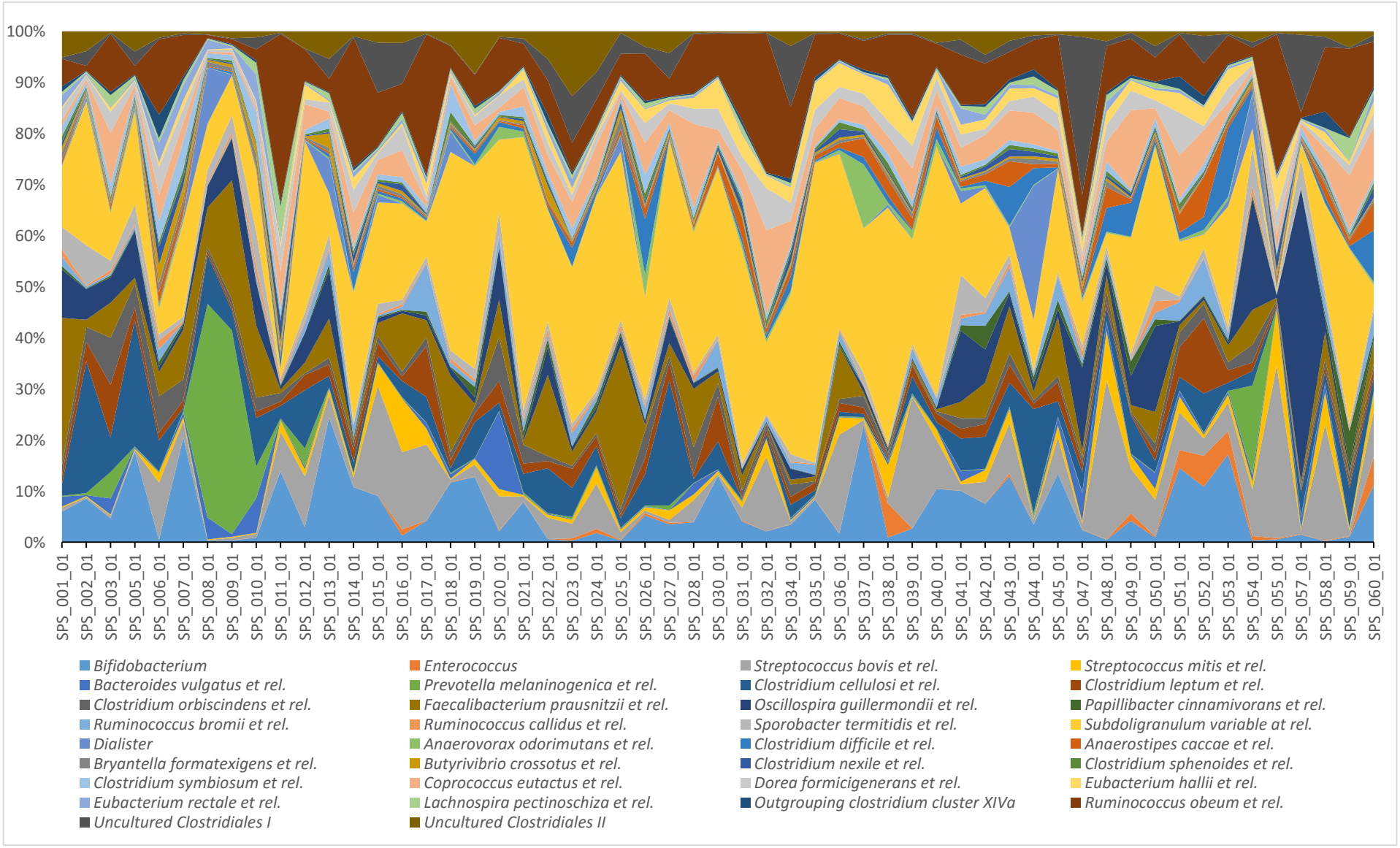
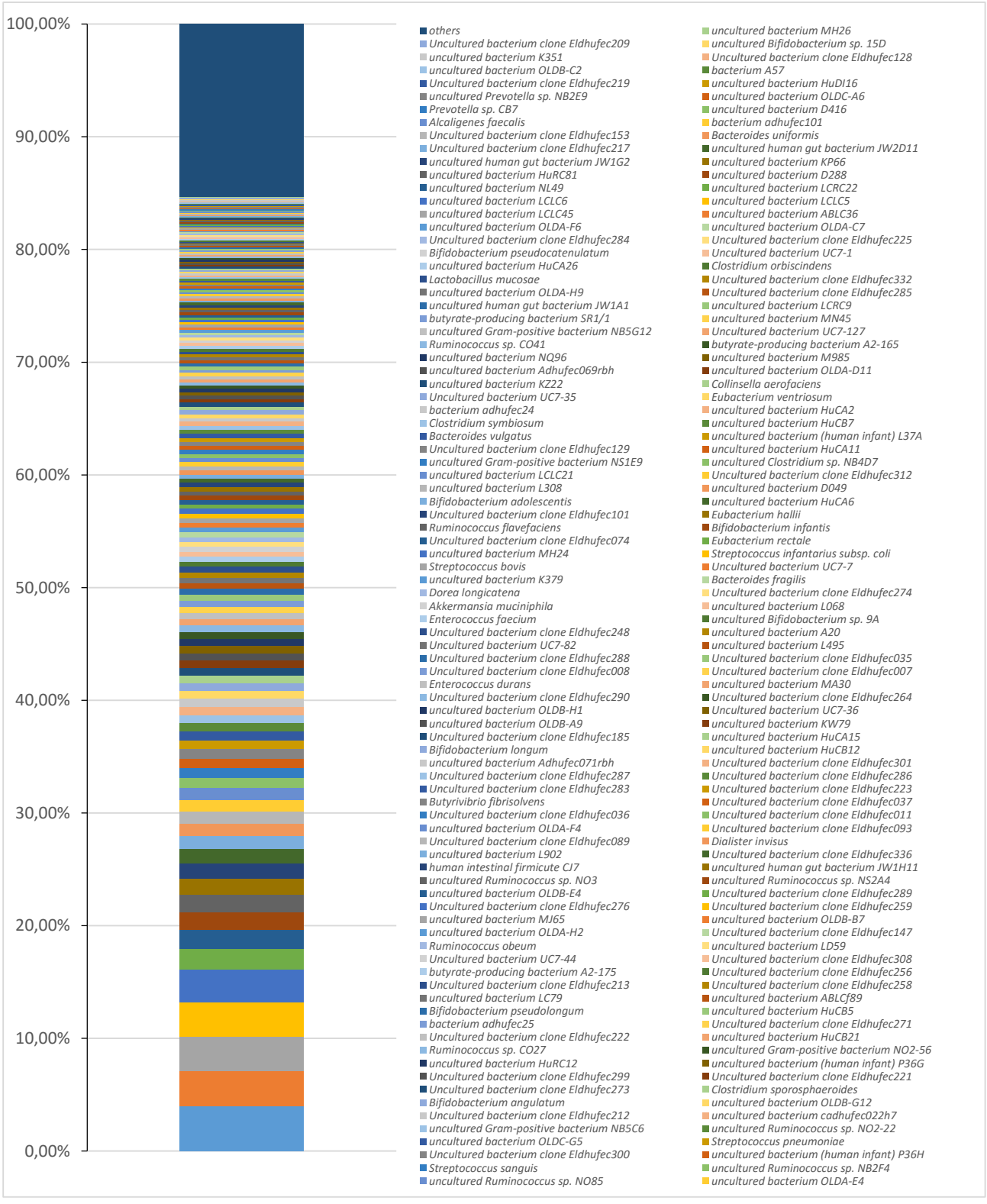


Figure 5.9 Relative contribution of species like level to the faecal microbiota of SNAS patients at baseline.



GI microbiota composition and its change during the trial

To obtain an overview of the similarity of the GI microbiota among all subjects over time, the pre-processed HITChip profiles were grouped using hierarchical clustering with correlation distance method (figure 5.10). Overall, high similarity among the microbiota profiles were found and samples did not cluster in a subject-wise manner. The largest differences in the microbiota profiles were inter-individual and inter-treatment. These evidences were confirmed when the microbiota profiles were compared at a higher resolution at the genus-like level (figure 5.11).

Figure 5.10 Hierarchical clustering of the HITChip profiles of SNAS patients investigated.

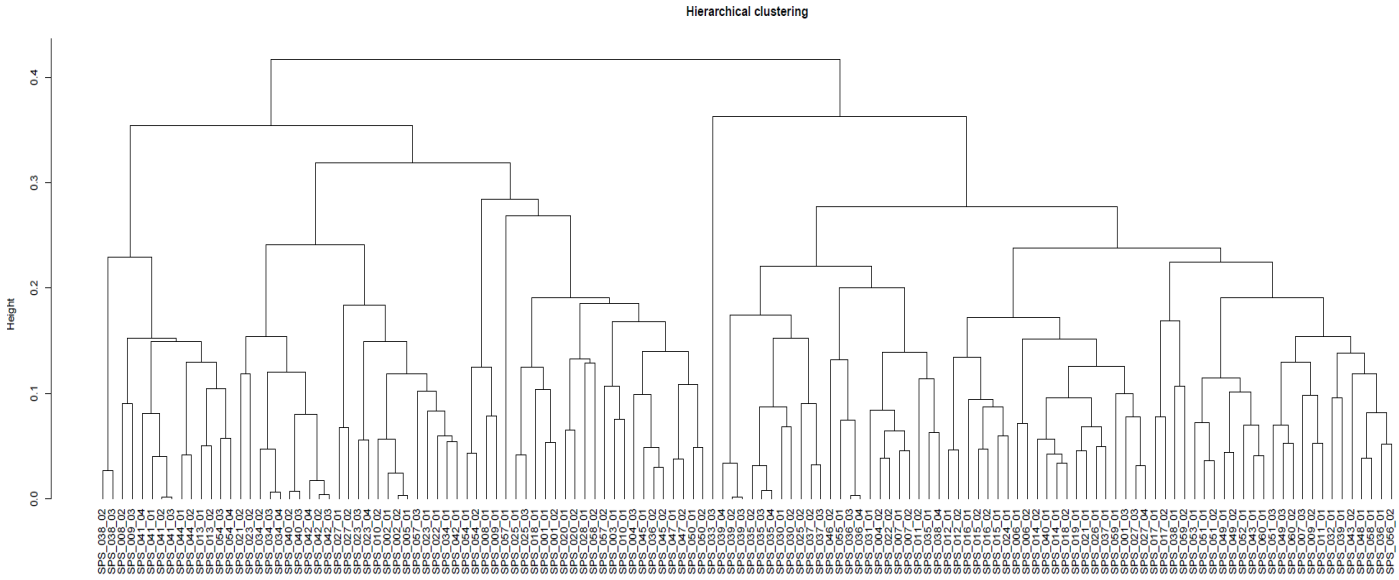
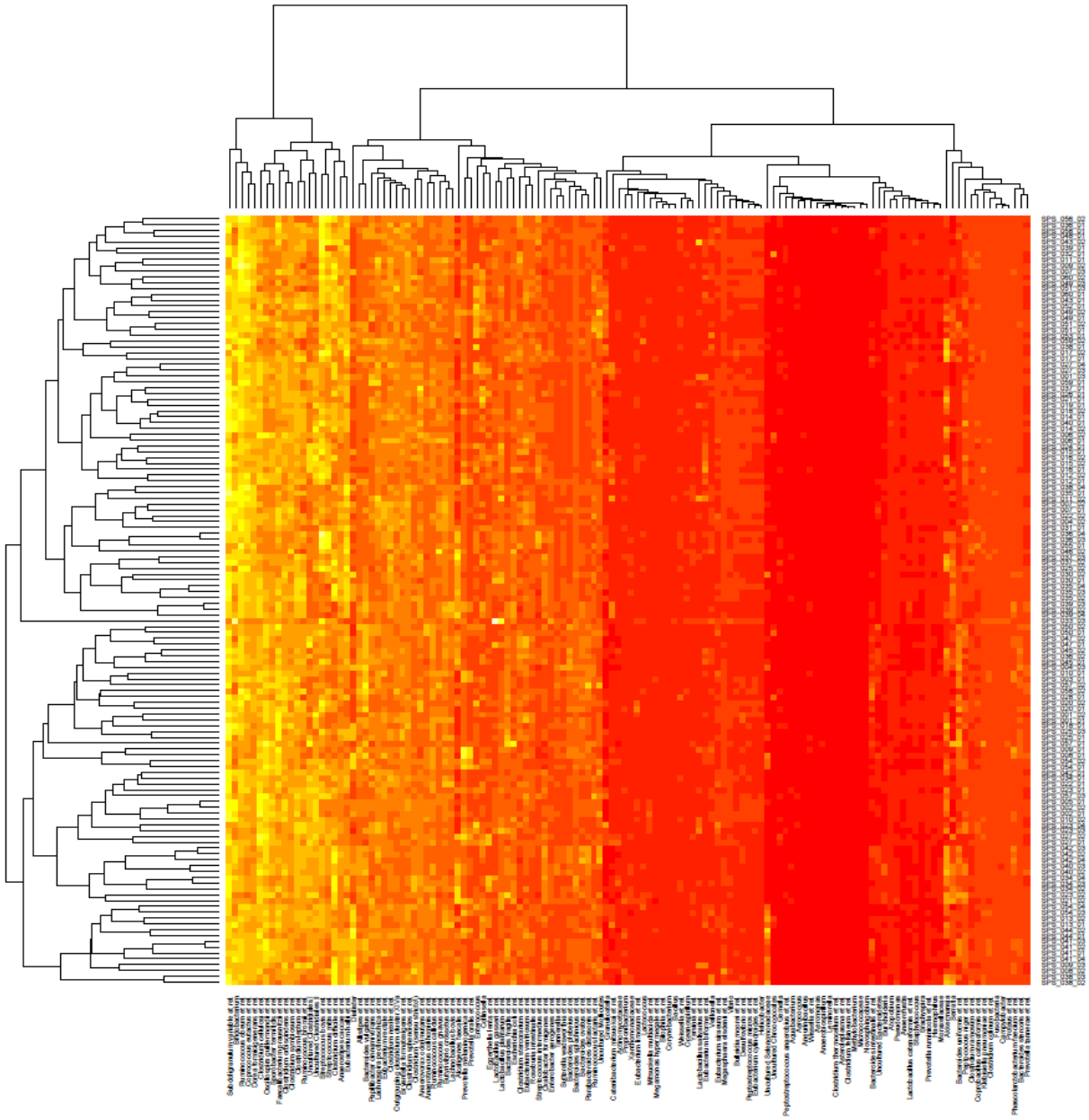


Figure 5.11 Heat map of relative abundance of bacterial groups at genus-like level.



Negative and positive correlations are indicated in red and yellow respectively.

Strong negatively correlation was found between all samples and genera *Gemella*, *Peptostreptococcus anaerobius et rel., Aquabacterium, Aerococcus, Aneurinibacillus, Wissella et rel., Aeromonas, Anaerobiospirillum, Leminorella, Clostridium thermocellum et rel.,*

Asteroleplasma et rel., Clostridium felsineum et rel., Methylobacterium, Micrococcaceae, Novosphingobium, Bacteroides intestinalis et rel., Uncultured Bacteroidetes, Burkholderia, Atopobium, Pseudomonas, Anaerofustis, Lactobacillus catenaformis et rel., Staphylococcus, Brachyspira, Prevotella ruminicola et rel., Haemophilus and *Moraxellaceae*. The genera previously reported exhibited irrelevant differences among patients and during the treatment. Differently, *Subdoligranulum variable at rel., Bifidobacterium, Ruminococcus obeum et rel., Coprococcus eutactus et rel., Dorea formicigenerans et rel., Clostridium cellulosi et rel., Oscillospira guillermondii et rel., Sporobacter termitidis et rel., Faecalibacterium prausnitzii et rel., Clostridium orbiscindens et rel., Clostridium symbiosum et rel., Clostridium leptum et rel., Ruminococcus bromii et rel., Uncultured Clostridiales I, Uncultured Clostridiales II, Streptococcus bovis et rel., Streptococcus mitis et rel., Clostridium difficile et rel., Anaerostipes caccae et rel., Eubacterium hallii et rel., Dialister, Allistipes et rel., Bacteroides vulgatus et rel., Papillibacter cinnamivorans et rel., Lachnospira pectinoschiza et rel.,* Eubacterium rectale et *rel., Clostridium nexile et rel., Outgrouping clostridium cluster XIVa, Bryantella formatexigens et rel., Clostridium sphenoides et rel., Clostridium \(sensu stricto\), Anaerovorax odorimutans et rel., Anaerotruncus colihominis et rel., Ruminococcus callidus et rel., Ruminococcus gnavus et rel., Butyrivibrio crossotus et rel., Lachnobacillus bovis et rel., Alcaligenes faecalis et rel., Prevotella melaninogenica et rel., Prevotella oralis et rel., Enterococcus, Collinsella, Eggerthella lenta et rel., Lactobacillus gasseri et rel., Lactobacillus plantarum et rel., Bacteroides fragilis et rel., Escherichia coli et rel., Clostridium stercorarium et rel., Eubacterium ventriosum et rel., Roseburia intestinalis et rel., Streptococcus intermedius et rel., Oxalobacter formigenes et rel., Enterobacter aerogenes et rel., Tannerella et rel., Sutterella wadsworthia et rel., Bacteroides plebeius et rel., Bacteroides splachnicus et rel., Bacteroides ovatus et rel., Parabacteroides distasonis et rel., Ruminococcus lactaris et rel.* and

Uncultured Mollicutes were characterized by a positive correlation and high changes among patients and sampling times.

The major differences in the microbiota composition were found in probiotic group not only during the treatment period (T3) but also at the end of the intervention (post-treatment T4). In order to investigate in more detail the changes occurred in microbiota composition, diversity (Inv Simpson and Shannon indices), richness and evenness were assessed. Generally, the diversity indices between baseline and post-probiotic treatment were significantly different ($p = 0.0161$) (figure 5.11). Moreover, differences were found in evenness comparing LGG treatment with both baseline ($p = 0.0153$) and diet ($p = 0.0432$) (figure 5.13). No significant differences were found in relation to the richness (figure 5.14).

Figure 5.12 Diversity score (Shannon and Inv Simposon indices) of SNAS patients at the different sampling times.

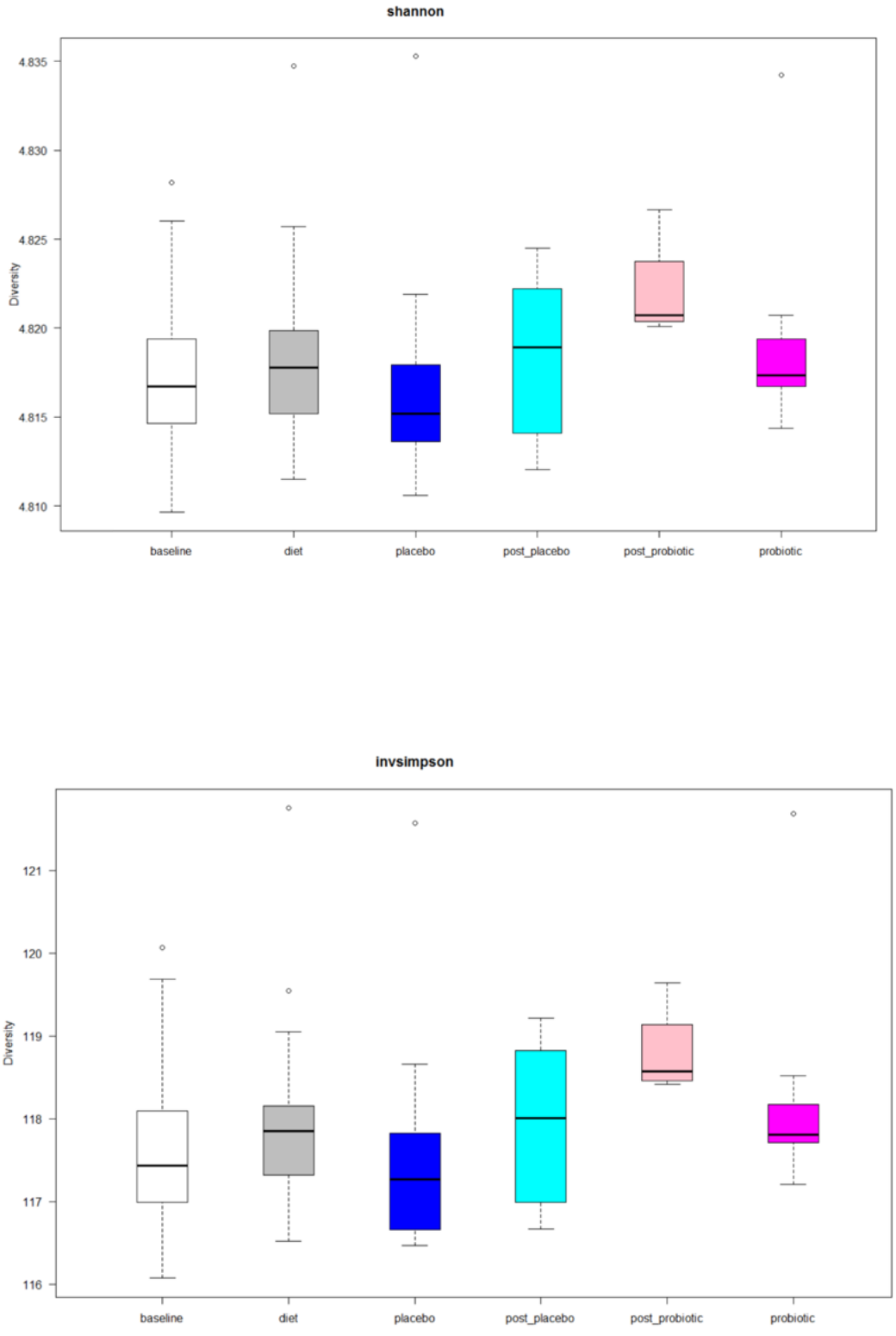


Figure 5.13 Evenness score of SNAS patients at the different sampling times.

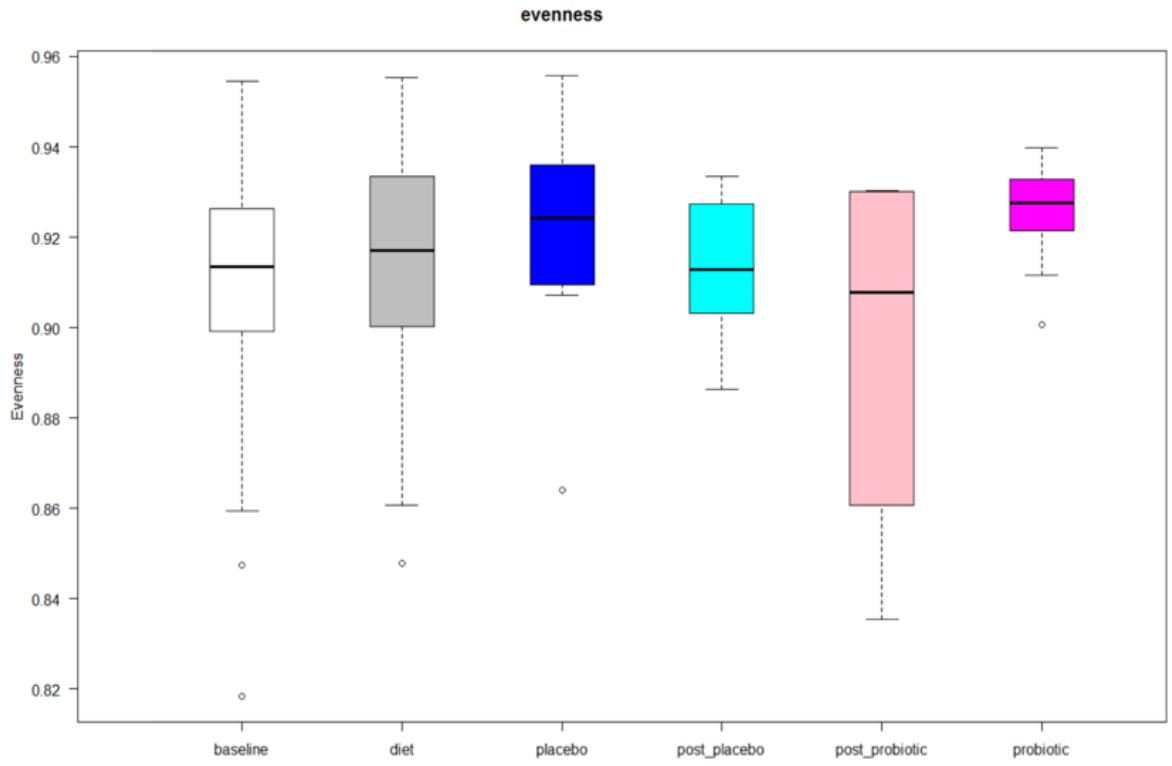
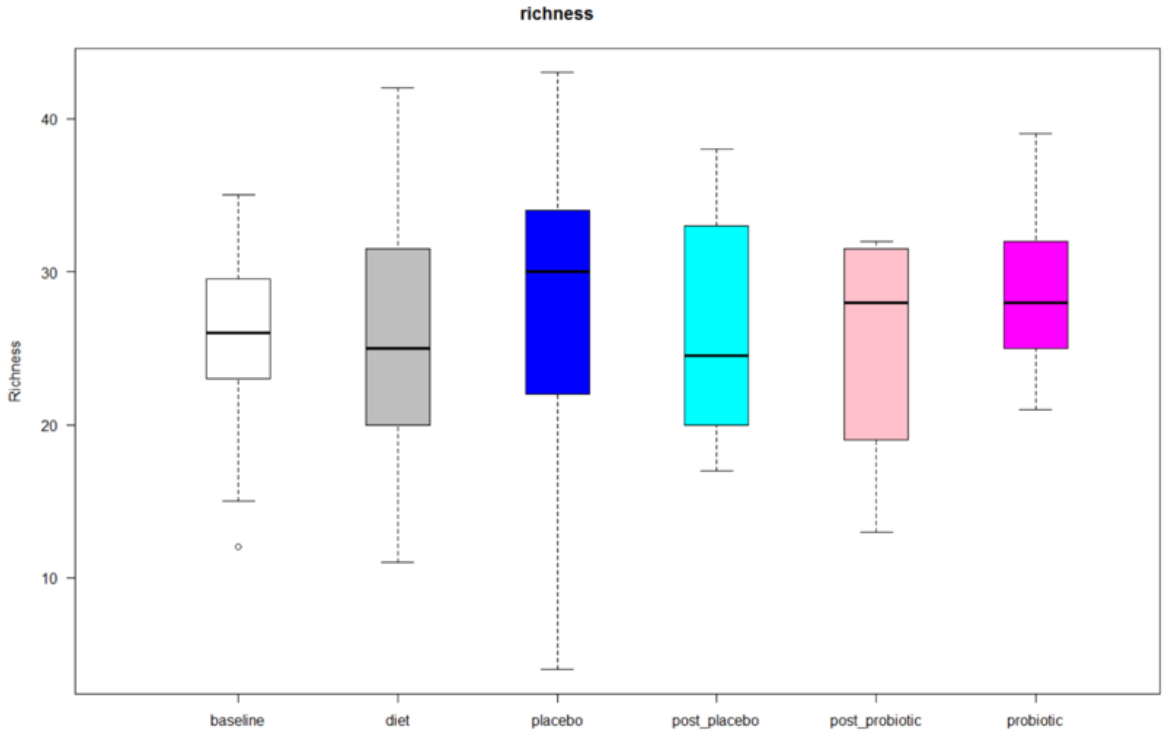
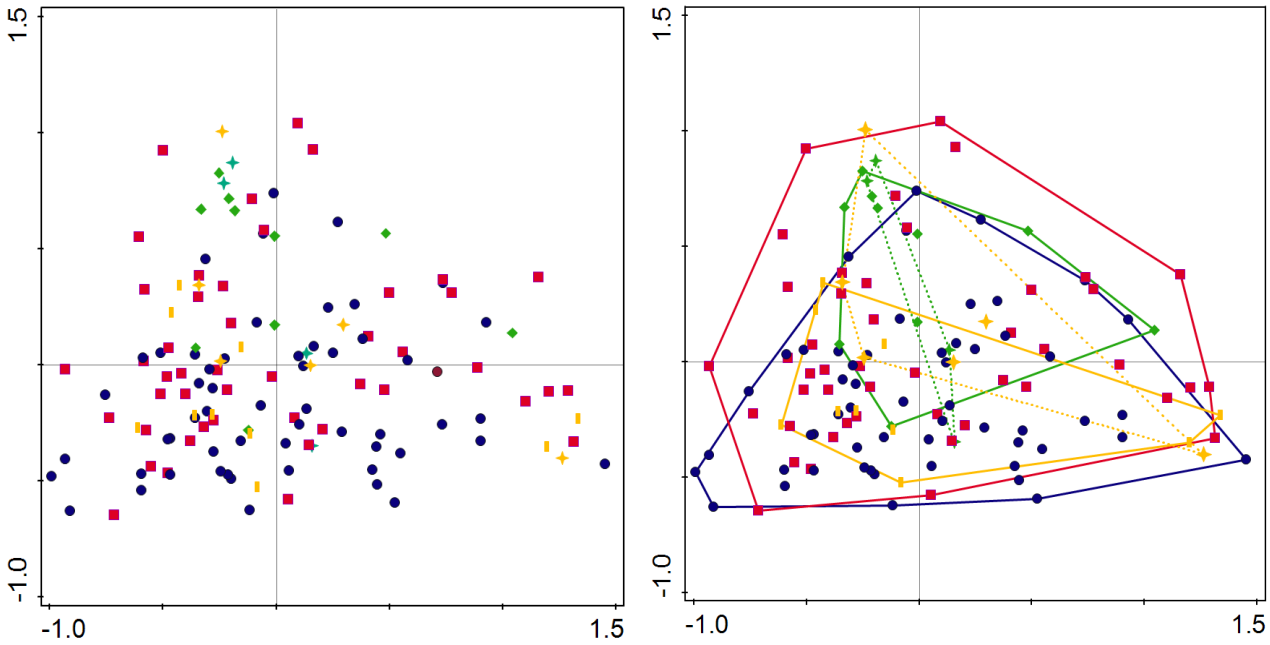


Figure 5.14 Richness score of SNAS patients at the different sampling times.



To relate changes in microbiota composition between patients groups and time of interventions, the hybridization signals of phylum and genus levels were subjected to Principal Component Analysis (PCA) and Redundancy Analysis (RDA). In the PCA of the phylum-like level microbiota profile (figure 5.15) and of genus-like level microbiota profile (figure 5.16), SNAS patients did not cluster separately in relation to different treatment and sampling time. However, the RDA of the phylum-like level microbiota profile (figure 5.17 a) and of genus-like level microbiota profile (figure 5.17 b) separated SNAS patients, in relation to the different sampling times, as judged by MCPP ($p=0.002$ phylum-like level; $p=0.01$ genus-like level). In detail, probiotic and post-probiotic treated samples clearly separated respect to baseline, diet, placebo and post-placebo treatments. The changes occurred in probiotic group were in deep studied performing the Wilcoxon rank sum test corrected for false discovery rate. In table 5.1 are reported the genera that showed statistically significant change.

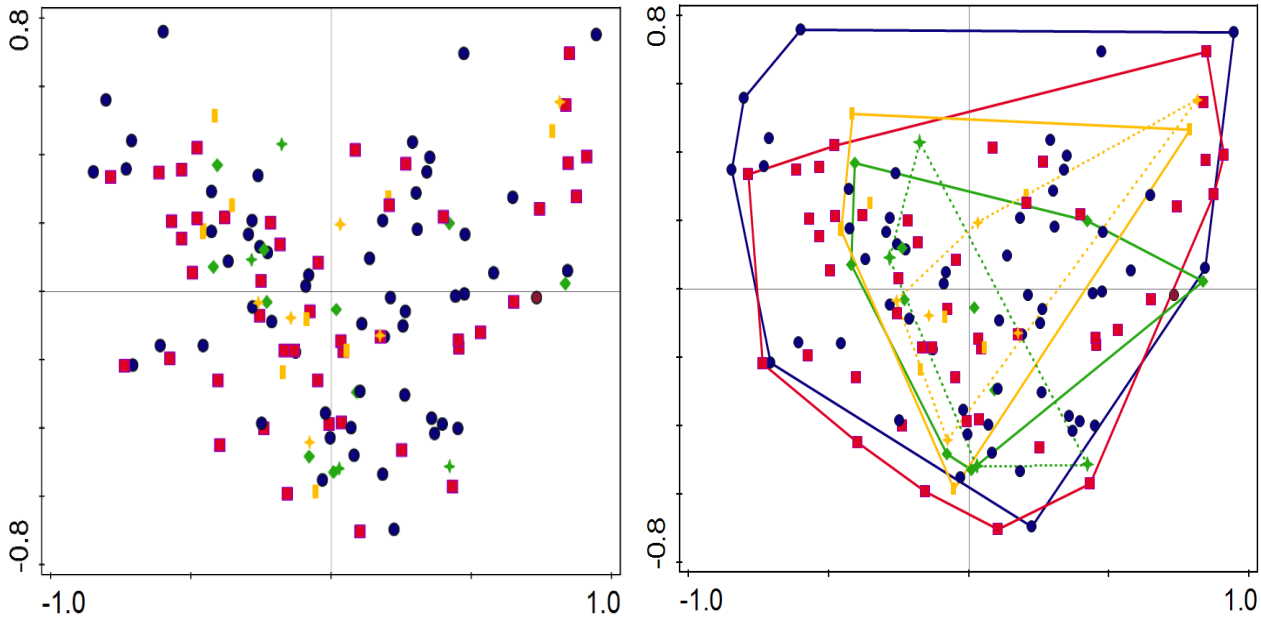
Figure 5.15 Principal component analysis (PCA) at phylum-like level of SNAS patients at different sampling times.



Log transformed data were used for analysis. In PCA the first two principal components capture 29.68% (PCA1) and 15.75% (PCA2) of variation respectively.

● Baseline (T1); ■ diet (T2); ◆ probiotic group (T3); ▮ placebo group (T3); ✦ probiotic group post-treatment (T4); ✦ placebo group post treatment (T4).

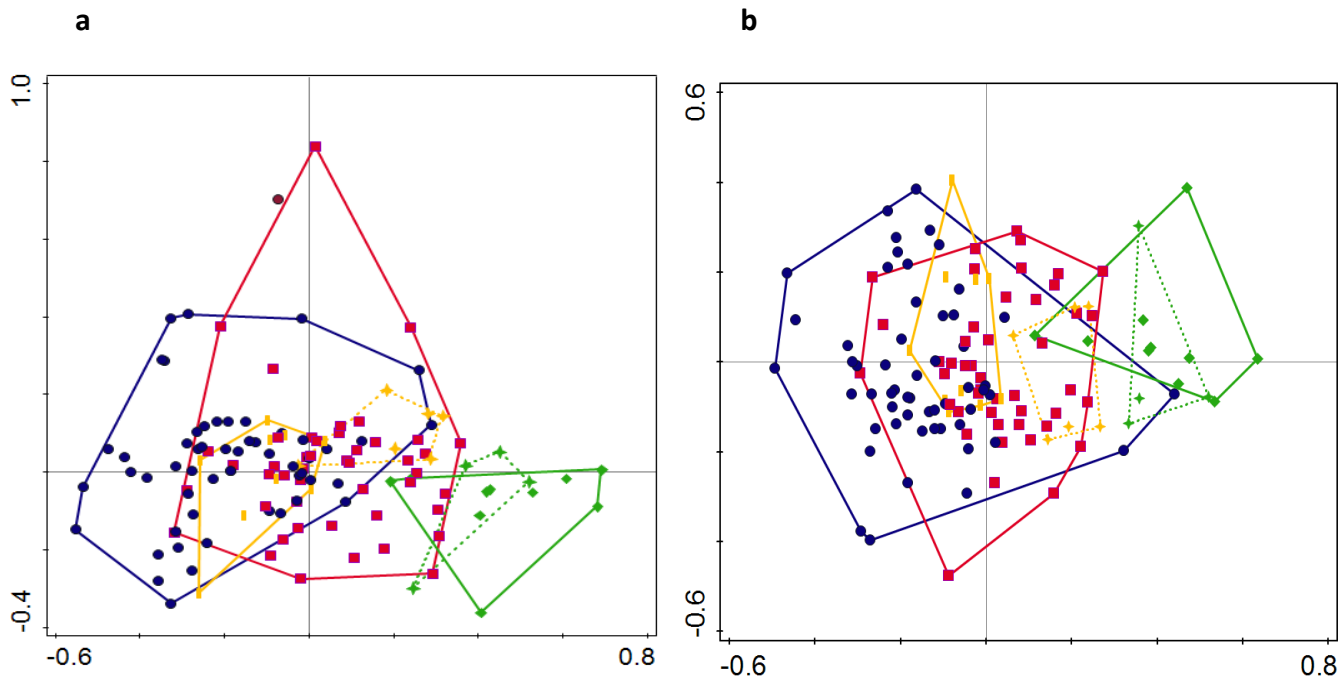
Figure 5.16 Principal component analysis (PCA) at genus-like level of SNAS patients at different sampling times.



Log transformed data were used for analysis. In PCA the first two principal components capture 19.108% (PCA1) and 9.84% (PCA2) of variation respectively.

Baseline (T1); diet (T2); probiotic group (T3); placebo group (T3); probiotic group post-treatment (T4); placebo group post treatment (T4).

Figure 5.17 Redundancy analysis (RDA) at phylum-like level (a) and genus-like level (b) of SNAS patients at different sampling times.



First and second ordination axes are plotted.

● Baseline (T1); ■ diet (T2); ◆ probiotic group (T3); ▮ placebo group (T3); ✦ probiotic group post-treatment (T4); ✦ placebo group post treatment (T4).

Table 5.1 Genera that that showed statistically significant change in probiotic group compared to baseline.

	pval
Clostridium difficile et rel.	0.003319001
Clostridium stercorarium et rel.	0.028323772
Clostridium \(sensu stricto\)	0.004864678
Lactobacillus salivarius et rel.	0.022788487
Lactobacillus plantarum et rel.	0.035966983
Escherichia coli et rel.	0.026046993
Uncultured Selenomonadaceae	0.048513735
Peptostreptococcus anaerobius et rel.	0.004864678
Pseudomonas	0.018190021
Lactobacillus gasseri et rel.	0.018576930
Yersinia et rel.	0.016998091
Mitsuokella multiacida et rel.	0.025587761
Lactococcus	0.032277639
Novosphingobium	0.018700819
Bacillus	0.010148672
Moraxellaceae	0.024812710
Leminorella	0.028323772
Peptostreptococcus micros et rel.	0.018190021
Staphylococcus	0.024812710
Micrococcaceae	0.018511513
Weissella et rel.	0.037935396
Eubacterium cylindroides et rel.	0.018511513
Atopobium	0.048513735
Clostridium colinum et rel.	0.038988400
Clostridium felsineum et rel.	0.010148672
Methylobacterium	0.007327700
Helicobacter	0.010148672
Anaerobiospirillum	0.028323772
Clostridium thermocellum et rel.	0.036928398
Fusobacteria	0.018511513
Haemophilus	0.028183679
Brachyspira	0.012651005
Bilophila et rel.	0.030738741
Megamonas hypermegale et rel.	0.018511513
Asteroleplasma et rel.	0.018511513
Proteus et rel.	0.033111597
Campylobacter	0.032277639
Oceanospirillum	0.025175553

DISCUSSION

The gut microbiota has gained importance in disease aetiology and pathology with emerging evidence demonstrating its key role in maintenance of health body, including protective, structural, and metabolic roles (6, 7). Nevertheless, several factors could modify the balance of gut microbiota in terms of alteration of its composition, change in bacterial metabolic activity and/or shift in local distribution of the community. Systemic Nickel Allergy Syndrome (SNAS) is an emergent pathological condition, rather than an occasional finding, related to the ingestion of Nickel containing food. This Syndrome has cutaneous and GI clinical manifestations that support the assumption that GI microbiota can be imbalanced. Up to now, no information is available concerning the intestinal microbiota composition of SNAS patients as well as about the possible usefulness of probiotic administration to improve symptoms and patient's quality of life. In the present study, the microbiota composition of SNAS patients was investigated, for the first time, using an advanced high throughput technique in order to assess the possible existence of a potential syndrome specific microbiota. In detail, by applying an highly reproducible phylogenetic microarray and taking into account subject specific variations it was possible to found that the faecal microbiota of SNAS patients is characterized by the dominance of the *Subdoligranulum variabile* and *Ruminococcus bromii* species. Both species belongs to the *Clostridium* cluster IV phylum and scientific information reported their presence, in high concentration, in IBS constipation patients. In fact, recent evidences (40) suggested that *R. bromii* is significantly more abundant in IBS patients with constipation symptoms than in healthy controls samples. *R. bromii* is a common starch degrader of the human intestinal microbiota (37) and the amount of this specie increase with a diet high in resistant starch (1). In the present study, the possible effect of diet could not be ruled out, but it is more likely that the slowed colonic transit, in some

SNAS patients, rather than a dietary effect, created a favourable environment for the *R. bromii*-like phylotype. In addition, the *Bacilli* phylum was represented almost exclusively by species member of *Streptococcus mitis* et rel. (*S. sanguis* and *S. pneumoniae*) and *Streptococcus bovis* et rel. (S*. salivarius, S. thermophiles, S. infantarium, and S. bovis*). Similarly to our results also in IBS patients high levels of *Streptococcus* spp. were found in faecal samples (74). Since *Streptococcus* spp. are among the dominant groups in the upper gastrointestinal tract, Rajilic (74) assessed that their presence at high levels in faecal samples implies the overall disturbance of the gastrointestinal microbiota in IBS patients. Moreover, SNAS patients presented the dominance of *Bacteroides fragilis* and *B. vulgatus.* As is known, among the species belonged to the *Bacteroidetes* phylum, *B. fragilis* is the most frequent isolate from clinical specimens and is regarded as the most virulent *Bacteroides*. The fimbriae and agglutinins of *B. fragilis* function as adhesins, allowing them to establish in the host tissue. The polysaccharide capsule, LPS, and a variety of enzymes protect *B. fragilis* from the host immune response. Recent studies indicate that *B. fragilis* may interfere with the peritoneal macrophages, the first host immunologic defence response to rupture of the intestine or other compromise of the peritoneal cavity (78, 81). Moreover, some studies have implicated *B. vulgatus* and *E. coli* in the development of Crohn's disease and ulcerative colitis (64, 4, 65). In addition, the microbiota characterization of SNAS patients highlighted the scarce occurrence of species commonly considered as "health- promoting" such us *Roseburia* spp. and *Feaealibacterium prausnitzii*. The latter belongs to the *Firmicutes* phylum and the *Clostridium leptum* group. The Meta-analysis of the Human Intestinal Tract project have shown that *F. prausnitzii* is one of the most abundant anaerobic bacteria in the human gut microbiota of healthy subjects and plays an important role in providing energy to the colonocytes and maintaining the intestinal health (39). Furthermore, there is emerging

laboratory evidence illustrating a strong anti-inflammatory effect of *F. prausnitzii* both *in vitro* and *in vivo*, and deficiency of this specie might provoke and enhance inflammation (47). The majority of recent studies find a higher rate of *F. prausnitzii* reduction in IBD patients compared to controls. Moreover, a recent meta-analysis suggested a possible link with the reduction of *F. prausnitzii* and the misbalance of the intestinal microbiota in IBD patients, especially CD patients with ileal involvement. A significant inverse correlation between disease activity and *F. prausnitzii* occurrence was also found in UC patients (41). In addition, similarly to UC also SNAS patients showed at baseline low abundance of the major butyrate producers, excepted for *Butyrivibrio fibrisolvens*. Another species, *Bilophila wadsworthia* more frequently detected among healthy subjects, occurred in SNAS patients in a very low amount (19, 26). Overall, our data suggest the alteration of the intestinal microbiota in SNAS patients and many likenesses with others pathological conditions related to gastrointestinal disorders.

In addition to the microbiota characterization, the present study was also conducted in order to investigate the effect of one-month low Ni diet. In fact, SNAS patients need to follow a lifelong Ni-poor diet, avoiding the majority of vegetables, which poses a potential risk for a deficit in essential elements and for dynamics of intestinal microbiota. Results showed that no statistical significant differences were found compared to baseline, in contrast with the alleviation of symptoms both cutaneous and gastro intestinal. This disagreement can be explained considering the short period of diet so further investigations using a prolonged observation period would be required. Differently, the administration of LGG during the desensitization period (NiTOH) was able to modify the microbiota composition. The changes occurred not only during the administration period but also during the post treatment. These evidences are in agreement with the results obtained in a previously study (56) conducted in

order to evaluate the therapeutic efficacy of the probiotic *Lactobacillus reuteri* DSM 17938 strain in patients suffering from SNAS, through the characterization of faecal lactic acid bacteria community and the identification of its shifts before and during the intervention. Based on the results *L. reuteri* administration was able to induce a significant decrease of clinical symptoms and a significant increase of LAB biodiversity and dynamics. Further study are necessary to in deep understand if the LGG administration alone can represent a valid treatment for the improvement of symptoms and the balance of intestinal microbiota.

REFERENCES

1. Abell G.C., Cooke C.M., Bennett C.N., Conlon M.A., Mc Orist A.L. (2008). Phylotypes related to *Ruminococcus bromii* are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. FEMS Microbiology Ecology, 66, 505-515.

2. Andrioli M., Trimboli P., Maio D., Persani L., Minelli M. (2015). Systemic nickel allergic syndrome as an immune-mediated disease with an increased risk for thyroid autoimmunity. Endocrine, 50, 807-810.

3. Arumugam M., Raes J., Pelletier E., Le Paslier D., Yamada T., Mende D.R. , Fernandes G.R. , Tap J., Bruls T., Batto J.M., Bertalan M., Borruel N., Casellas F., Fernandez L., Gautier L., Hansen T., Hattori M., Hayashi T., Kleerebezem M., Kurokawa K., Leclerc M., Levenez F., Manichanh C., Nielsen H.B., Nielsen T., Pons N., Poulain J., Qin J., Sicheritz-Ponten T., Tims S., Torrents D., Ugarte E. , Zoetendal E.G., Wang J., Guarner F., Pedersen O., de Vos W.M., Brunak S., Dore´ J., MetaHIT Consortium, Weissenbach J., Ehrlich D., Bork P. (2011). Enterotypes of the human gut microbiome. Nature, 473, 174-180.

4. Bamias G., Okazawa A., Rivera-Nieves J., Arseneau K.O., De La Rue S.A., Pizarro T.T., Cominelli F. (2007). Commensal bacteria exacerbate intestinal inflammation but are not essential for the development of murine ileitis. Journal of Immunology, 178, 1809–1818.

5. Benson A.K., Kelly S.A., Legge R., Ma F.R., Low S.J., Kim J., Zhang M., Oh P.L., Nehrenberg D., Hua K.J., Kachmanc S.D., Moriyamad E.N., Waltera J., Petersona D.A., Pomp D. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proceedings of the National Academy of Sciences of the United States of America, 107, 18933-18938.

6. Cani P.D. (2009). The role of the gut microbiota in energy metabolism and metabolic disease. Current Pharmaceutical Design, 15, 1546-1558.

7. Cani P.D., Delzenne N.M. (2009). Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Current Opinion in Pharmacology, 9, 737-743.

8. Cazzato I.A., Vadrucci E., Cammarota G., Minelli M., Gasbarrini A. (2011). Lactose intolerance in systemic nickel allergy syndrome. International Journal of Immunopathology and Pharmacology, 24, 535–537.

9. Centanni M., Turroni S., Biagi E., Severgnini M., Consolandi C., Brigidi P., Candela M. (2013). A novel combined approach based on HTF-Microbi.Array and qPCR for a reliable

characterization of the *Bifidobacterium* dominated gut microbiota of breast-fed infants. FEMS Microbiology Letters, 343, 121-126.

10. Christensen O.B., Möller H. (1975). External and internal exposure to the antigen in the hand eczema of nickel allergy. Contact Dermatitis, 1, 136-141.

11. Cirla A.M., Cirla P.E. (2012). Nickel dermatitis, systemic nickel allergy syndrome, immuno-genesis, immune-tolerance: an Italian study. Giornale italiano di medicina del lavoro ed ergonomia, 34, 147–149.

12. Claesson M.J., Cusack S., O'Sullivan O., Greene-Diniz R., de Weerd H., Flannery E., Marchesi J.R., Falush D., Dinan T., Fitzgerald G., Stanton C., van Sinderen D., O'Connor M., Harnedy N., O'Connor K., Henry C., O'Mahony D., Fitzgerald A.P., Shanahan F., Twomey C., Hill C., Ross R.P., O'Toole P.W. (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proceedings of the National Academy of Sciences of the United States of America, 108, 4586-4591.

13. Costello E.K., Lauber C.L., Hamady M., Fierer N., Gordon J.I., Knight R. (2009). Bacterial community variation in human body habitats across space and time. Science 326, 1694-1697.

14. Delgado S., Suarez A., Mayo B. (2006). Identification of dominant bacteria in feces and colonic mucosa from healthy Spanish adults by culturing and by 16S rDNA sequence analysis. Digestive Disease and Sciences, 51, 744-751.

15. Dethlefsen L., McFall-Ngai M., Relman D.A. (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature, 449, 811-818.

16. Di Gioacchino M., Boscolo P., Cavallucci E., Verna N., Di Stefano F., Di Sciascio M., Masci S., Andreassi M., Sabbioni E., Angelucci D., Conti P. (2000). Lymphocyte subset changes in blood and gastrointestinal mucosa after oral nickel challenge in nickel-sensitized women. Contact Dermatitis, 43, 206-211.

17. Di Gioacchino M., Ricciardi L., De Pità O., Minelli M., Patella V., Voltolini S., Di Rienzo V., Braga M., Ballone E., Mangifesta R., Schiavino D. (2014). Nickel oral hyposensitization in patients with systemic nickel allergy syndrome. Annals of medicine, 46, 31–37.

18. Duntas L.H. (2008). Environmental factors and autoimmune thyroiditis. Nature clinical practice. Endocrinology & Metabolism, 4, 454–460.

19. Eckburg P.B., Bik E.M., Bernstein C.N., Purdom E., Dethlefsen L., Sargent M., Gill S.R., Nelson K.E., Relman D.A. (2005) Diversity of the human intestinal microbial flora. Science, 308, 1635-1638.

20. Faith J.J., Guruge J.L., Charbonneau M., Subramanian S., Seedorf H., Goodman A.L., Clemente J.C., Knight R., Heath A.C., Leibel R.L., Rosenbaum M., Gordon J.I. (2013). The long-term stability of the human gut microbiota. Science, 341, DOI: 10.1126/science.1237439.

21. Feng M., Zhang F.B., Deng H.R. (2013). The CTLA4 +49A/G polymorphism is associated with an increased risk of Hashimoto's thyroiditis in Asian but not Caucasian populations: an updated meta-analysis. Endocrine 44, 350–358.

22. Finegold S.M. (2008). Therapy and epidemiology of autism-clostridial spores as key elements. Medical Hypotheses, 70, 508-511.

23. Flores G.E., Caporaso J.G., Henley J.B., Rideout J.R., Domogala D., Chase J., Leff J.W., Vazquez-Baeza Y., Gonzalez A., Knight R., Dunn R.R., Fierer N. (2014). Temporal variability is a personalized feature of the human microbiome. Genome Biology, 15, 1.-13.

24. Fraher M.H., O'Toole P.W., Quigley E.M. (2012). Techniques used to characterize the gut microbiota: A guide for the clinician. Nature reviews Gastroenterology & hepatology, 9, 312-322.

25. Gangemi S., Ricciardi L., Minciullo P.L., Cristani M., Saitta S., Chirafisi J., Spatari G., Santoro G., Saija A. (2009). Serum levels of protein oxidation products in patients with nickel allergy. Allergy and Asthma Proceedings, 30, 552–557.

26. Hayashi H., Sakamoto M., Benno Y. (2002) Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiology and Immunology, 46, 535-548.

27. Hill M. (1973). Diversity and evenness: a unifying notion and its consequences. Ecology, 54, 427-432.

28. Holling, S.C. (1973). Resilience and Stability of Ecological Systems. Annual Review of Ecology and Systematics, 4, 1-23.

29. Holmes E., Loo R.L., Stamler J., Bictash M., Yap I.K., Chan Q., Ebbels T., De Iorio M., Brown I.J., Veselkov K.A., Daviglus M.L., Kesteloot H., Ueshima H., Zhao L., Nicholson J.K., Elliott P. (2008). Human metabolic phenotype diversity and its association with diet and blood pressure. Nature, 453, 396-400.

30. Huse S.M., Dethlefsen L., Huber J.A., Welch D.M., Relman D.A., Sogin M.L. (2008). Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. PLoS Genetics 4, 1-10.

31. Huttenhower C., Gevers D., Knight R., Abubucker S., Badger J.H., Chinwalla A.T., Creasy H.H., Earl A.M., FitzGerald M.G., Fulton R.S*.,* et al. (2012). Structure, function and diversity of the healthy human microbiome. Nature *486*, 207-214.

32. Kashyap P.C., Marcobal A., Ursell, L.K., Larauche, M., Duboc, H., Earle, K.A., Sonnenburg, E.D., Ferreyra, J.A., Higginbottom, S.K., Million, M.*,* Million M., Tache Y., Pasricha P.J., Knight R., Farrugia G., Sonnenburg J.L. (2013). Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. Gastroenterology 144, 967-977.

33. Kashyap P.C., Marcobal A., Ursell L.K., Smits S.A., Sonnenburg E.D., Costello E.K., Higginbottom S.K., Domino S.E., Holmes S.P., Relman D.A*.,* Knightc R., Gordonh J.I., Sonnenburg J.L. (2013). Genetically dictated change in host mucus carbohydrate landscape exerts a diet-dependent effect on the gut microbiota. Proceedings of the National Academy of Sciences of the United States of America 110, 17059-17064.

34. Kinross J.M., Darzi A.W., Nicholson J.K. (2011). Gut microbiome-host interactions in health and disease. Genome Medicine, 3, 2-12.

35. Krogius-Kurikka L., Lyra A., Malinen E., Aarnikunnas J., Tuimala J., Paulin L., Makivuokko H., Kajander K., Palva A. (2009). Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterology 9, 95.

36. Le Chatelier E., Nielsen T., Qin J., Prifti E., Hildebrand F., Falony G., Almeida M., Arumugam M., Batto J.M., Kennedy S., Leonard P., Li J., Burgdorf K.,Grarup N., Jørgensen T., Brandslund I.,+ Nielsen H.B., Juncker A.S., Bertalan M., Levenez F., Pons N., Rasmussen S., Sunagawa S., Tap J., Tims S.,Zoetendal E.G., Brunak S., Clément K., Doré J., Kleerebezem M., Kristiansen K., Renault P., Sicheritz-Ponten T., de Vos W.M., Zucker J.D., Raes J.,Hansen T., MetaHIT consortium, Bork P., Wang J., Ehrlich S.D., Pedersen O. (2013). Richness of human gut microbiome correlates with metabolic markers. Nature, 500, 541-546.

37. Leitch E.C., Walker A.W., Duncan S.H., Holtrop G., Flint HJ. (2007). Selective colonization of insoluble substrates by human faecal bacteria. Environmental Microbiology, 9, 667-679.

38. Li J, Jia H., Cai X., Zhong H., Feng Q., Sunagawa S., Arumugam M., Kultima J.R., Prifti E., Nielsen T., Juncker A.S., Manichanh C., Chen B., Zhang W., Levenez F., Wang J., Xu X., Xiao L., Liang S., Zhang D., Zhang Z., Chen W., Zhao H., Al-Aama J.Y., Edris S.,Yang H., Wang J., Hansen T., Nielsen H.B., Brunak S., Kristiansen K., Guarner F., Pedersen O., Doré J., Ehrlich S.D., MetaHIT Consortium, Bork P., Wang J. (2014). An integrated catalog of reference genes in the human gut microbiome. Nature Biotechnology, 32, 834-841.

39. Louis P., Flint H.J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiology Letters, 294, 1–8.

40. Lyra A., Rinttilä T., Nikkilä J., Krogius-Kurikka L., Kajander K., Malinen E., Mättö J., Mäkelä L., Palva A. (2009). Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. World Journal of Gastroenterology, 15, 5936-5945.

41. Machiels K., Joossens M., Sabino J. De Preter V., Arijs I., Eeckhaut V., Ballet V., Claes K., Van Immerseel F., Verbeke K., Ferrante M.,Verhaegen J., Rutgeerts P., Vermeire S. (2013). A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. Gut, 63, 1275-83.

42. Marchesi J.R., Holmes E., Khan F., Kochhar S., Scanlan P., Shanahan F., Wilson I.D., Wang Y. (2007). Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. Journal of proteome research, 6, 546-551.

43. Martinez I., Muller C.E., Walter J. (2013). Long-term temporal analysis of the human fecal microbiota revealed a stable core of dominant bacterial species. PLoS ONE 8, e69621.

44. Maukonen J., Matto J., Satokari R., Saderlund H., Mattila-Sandholm T., Saarela M. (2006). PCR DGGE and RT-PCR DGGE show diversity and short-term temporal stability in the Clostridium coccoides- *Eubacterium rectale* group in the human intestinal microbiota. FEMS Microbiology Ecology 58, 517-528.

45. Maukonen J., Simoes C., Saarela M. (2012). The currently used commercial DNA extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. FEMS microbiology ecology, 79, 697-708.

46. Minelli M., Schiavino D., Musca F., Bruno M.E., Falagiani P., Mistrello G., Riva G., Braga M., Turi M.C., Di Rienzo V., Petrarca C., Schiavone C., Di Gioacchino M. (2010). Oral hyposensitization to nickel induces clinical improvement and a decrease in TH1 and TH2 cytokines in patients

with systemic nickel allergy syndrome. International Journal of Immunopathology and Pharmacology, 23, 193-201.

47. Packey C.D., Sartor R.B. (2009). Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Current Opinion in Infectious Diseases, 22, 292–301.

48. Paliy O., Kenche H., Abernathy F., Michail S. (2009). High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. Applied Environmental Microbiology, 75, 3572-3579.

49. Pizzutelli S. (2011). Systemic nickel hypersensitivity and diet: myth or reality? European annals of allergy and clinical immunology, 43, 5-18.

50. Qin J., Li R., Raes J., Arumugam M., Burgdorf K.S., Chaysavanh M., Trine N., Pons N., Levenez F., amada T., Mende D.R., Li J., Xu J., Li S., Li D., Cao J., Wang B., Liang H., Zheng H., Xie Y., Tap J., Lepage P., Bertalan M., Batto J.M., Hansen T., Le Paslier D., Linneberg A., Nielsen H.B, Pelletier E., Renault P., Sicheritz-Ponten T., Turner K., Zhu H., Yu C., Li S., Min J., Zhou Y., Li Y., Zhang X., Songgang L., Qin N, Yang H, Wang J., Brunak S., Doré J., Guarner F., Kristiansen K., Pedersen O., Parkhill J., eissenbach J.W.,MetaHIT Consortium, Bork P., S. Ehrlich D., Wang J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59-65.

51. Rajilic-Stojanovic M., Smidt H., de Vos W.M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. Environmental Microbiology 9, 2125-2136.

52. Rajilić-Stojanović M., Heilig H., Molenaar D., Kajander K., Smidt H., de Vos W.M. (2009). Development and application of the human intestinal tract chip (HITChip), a phylogenetic microarray: Absence of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environmental Microbiology, 11, 1736-1743.

53. Rajilic-Stojanovic M., Heilig H.G., Molenaar D., Kajander K., Surakka A., Smidt H., de Vos W.M. (2009). Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environmental Microbiology, 11, 1736-1751.

54. Rajilic-Stojanovic M., Heilig H.G., Tims S., Zoetendal E.G., de Vos W.M. (2012). Long-term monitoring of the human intestinal microbiota composition. Environmental Microbiology, 15, 1146–1159.

55. Rajilic-Stojanovic M., de Vos W.M. (2014). The first 1000 cultured species of the human gastrointestinal microbiota. FEMS microbiology reviews, 38, 996-1047.

56. Randazzo C.L., Pino A., Ricciardi L., Romano C., Comito D., Arena E., Saitta S., Caggia C. (2014). Probiotic supplementation in Systemic Nickel Allergy Syndrome patients: study of its effects on lactic acid bacteria population and on clinical symptoms. Journal of applied microbiology, 118, 202-211.

57. Ricciardi L., Arena A., Arena E., Zambito M., Ingrassia A., Valenti G., Loschiavo G., D'Angelo A., Saitta S. (2014). Systemic nickel allergy syndrome: epidemiological data from four Italian allergy units. International Journal of Immunopathology and Pharmacology, 27, 131–136.

58. Rosato E., Giovannetti A., Rossi C., Menghi G., Pisarri S., Salsano F. (2009). Recurrent infections in patients with nickel allergic hypersensitivity. Journal of biological regulators and homeostatic agents, 23, 173-80.

59. Rosato E., Carello R., Gabriele I., Molinaro I., Galli E., Salsano F. (2011). Recurrent infections in children with nickel allergic contact dermatitis. Journal of biological regulators and homeostatic agents, 25, 661-665.

60. Salonen A., Nikkila J., Jalanka-Touvinen J., Immonen O., Rajilic-Stojanovic, M. (2010). Comparative analysis of faecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. Journal of microbialogical Methods, 81, 127-134.

61. Salonen A., Salojarvi J., Lahti L., de Vos W.M. (2012). The adult intestinal core microbiota is determined by analysis depth and health status. Clinical Microbiology and Infection 18, 16-20.

62. Scanlan P.D., Shanahan F., O'Mahony C., Marchesi J.R. (2006). Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. Journal of Clinical Microbiology, 44, 3980-3988.

63. Simpson E. (1949). Measurements of diversity. Nature, 163.

64. Strober W. (2006). Unraveling gut inflammation. Science, 313, 1052–1054.

65. Strober W., Fuss I., Mannon P. (2007). The fundamental basis of inflammatory bowel disease. Journal of Clinical Investigation, 117, 514–521.

66. Swidsinski A., Loening-Baucke V., Lochs H. (2005). Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World Journal of Gastroenterology, 11, 1131-1140.

67. Tannock G.W., Munro K., Harmsen H.J., Welling G.W., Smart J., Gopal P.K. (2000). Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Applied and Environmental Microbiology, 66, 2578-2588.

68. Tap J., Mondot S., Levenez F., Pelletier E., Caron C., Furet J.P., Ugarte E., Muñoz-Tamayo R., Paslier D.L., Nalin R., Dore J., Leclerc M. (2009). Towards the human intestinal microbiota phylogenetic core. Environmental microbiology, 11, 2574-2584.

69. Thyssen J.P., Linneberg A., Menné T., Johansen J.D. (2007). The epidemiology of contact allergy in the general population – prevalence and main findings. Contact Dermatitis, 57, 287–299.

70. Tims S., Derom C., Jonkers D.M., Vlietinck R., Saris W.H., Kleerebezem M., de Vos W.M., Zoetendal E.G. (2013). Microbiota conservation and BMI signatures in adult monozygotic twins. ISME Journal, 7, 707-717.

71. Tottey W., Denonfoux J., Jaziri F., Parisot N., Missaoui M., Hill D., Borrel G., Peyretaillade E., Alric M., Harris H.M., Jeffery I.B., Claesson M.J., O'Toole P.W., Peyret P., Brugère J.F. (2013). The human gut chip "HuGChip", an explorative phylogenetic microarray for determining gut microbiome diversity at family level. PLoS One, 8, e62544.

72. Turnbaugh P.J., Ley R.E., Mahowald M.A., Magrini V., Mardis E.R., Gordon J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, 444, 1027-1031.

73. Turnbaugh P.J., Hamady M., Yatsunenko T., Cantarel B.L., Duncan A., Ley R.E., Sogin M.L., Jones W.J., Roe B.A., Affourtit J.P., Egholm M., Henrissat B., Heath A.C., Knight R., Gordon J.I. (2009). A core gut microbiome in obese and lean twins. Nature, 457, 480-484.

74. T Rajilic-Stojanovic M. (2007). Diversity of the human gastrointestinal microbiota, novel perspectives from high throughput analyses. PhD thesis, Wageningen University, The Netherlands.

75. Van Ginneken C. (2006). Gastrointestinal Tract. In Understanding Drug Release and Absorption Mechanisms. A Physical and Mathematical Approach. CRC Press, 211-230.

76. Vanhoutte T., Huys G., Brandt E., Swings J. (2004). Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiology Ecology, 48, 437-446.

77. Vaughan E.E., de Vries M.C., Zoetendal E.G., Ben-Amor K., Akkermans A.D., de Vos W.M. (2002). The intestinal LABs. Antonie Van Leeuwenhoek, 82, 341-352.

78. Vieira J.M., Vallim D.C., Ferreira E.O., Seabra S.H., Vommaro R.C., Avelar K.E., De S.W., Ferreira M.C., Domingues R.M. (2005). *Bacteroides fragilis* interferes with iNOS activity and leads to pore formation in macrophage surface. Biochemical and biophysical research communications, 326, 607–613.

79. Walker A.W., Duncan S.H., Louis P., Flint H.J. (2014). Phylogeny, culturing, and metagenomics of the human gut microbiota. Trends in Microbiology, 22, 267-274.

80. Werner J.J., Zhou D., Caporaso J.G., Knight R., Angenent L.T. (2012). Comparison of illumine paired-end and single-direction sequencing for microbial 16S rRNA gene amplicon surveys. ISME Journal, 6, 1273-1276.

81. Wexler Hannah M. (2007). *Bacteroides*: the Good, the Bad, and the Nitty-Gritty. Clinical Microbiology Reviews, 593–621.

82. Yatsunenko T., Rey F.E., Manary M.J., Trehan I., Dominguez-Bello M.G., Contreras M., Magris M., Hidalgo G., Baldassano R.N., Anokhin A.P., Heath A.C., Warner B., Reeder J., Kuczynski J., Caporaso J.G., Lozupone C.A., Lauber C., Clemente J.C., Knights D., Knight R., Gordon J.I. (2012). Human gut microbiome viewed across age and geography. Nature, 486, 222-227.

83. Zoetendal E.G., Akkermans A.D., De Vos W.M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Applied and Environmental Microbiology 64, 3854-3859.

84. Zoetendal E.G., Rajilic-Stojanovic M., de Vos W.M. (2008). High throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut, 57, 1605-1615

Appendices

LIST OF FIGURES

LIST OF TABLES

LIST OF PUBBLICATIONS

Randazzo C.L., Pino A., Ricciardi L., Romano C., Comito D., Arena E., Saitta S., Caggia C. (2014). Probiotic supplementation in Systemic Nickel Allergy Syndrome patients: study of its effects on lactic acid bacteria population and on clinical symptoms. Journal of applied microbiology, 118, 202-211.

C.L. Randazzo, A. Todaro, A. Pino, I. Pitino, O. Corona, A. Mazzaglia, C. Caggia. Giarraffa and Grossa di Spagna naturally fermented table olives: Effect of starter and probiotic cultures on chemical, Microbiological and sensory traits Food Research International 62 (2014) 1154–1164.

Caggia C., De Angelis M., Pitino I., Pino A., Randazzo C.L. Probiotic features of Lactobacillus strains isolated from Ragusano and Pecorino Siciliano cheeses. Food Microbiology. Food Microbiology 50, 109-117.

Randazzo C.L., Todaro A., Pino A., Pitino I., Corona O., De Angelis M., Caggia C. Microbiota and metabolome of controlled and spontaneous fermentation of Nocellara etnea table olives. Submitted for publication.

Randazzo C.L., Pino A., Russo N., Van Hoorde K., Carpino S., Caggia C. *Lactobacillus rhamnosus* H25 and *Lactobacillus paracasei* N24 for probiotic Pecorino Siciliano cheese production: study of probiotics survival during production, ripening and through the human gastrointestinal tract. Manuscript in preparation.

Pino A., Randazzo C.L., Cianci A., Bartolo E., Caggia C. Effect of *Lactobacillu rhamnosus* GG and Lactoferrin administration on vaginal microbiota of women with bacterial vaginosis. Manuscript in preparation.

Pino A., Randazzo C.L., Zoetendal W., de Vos W. Intestinal microbiota composition in Systemic Nickel Allergy Syndrome (SNAS) patients: a high throughput microarray analysis. Manuscript in preparation.

Carpino C., Randazzo C.L., De Angelis M., Pino A., Russo N., Rapisarda T., Belvedere G., Caggia C. influence of Ragusano cheese biofilm microbiota on flavour compounds formation. Submitted for pubblication

POSTER PRESENTATIONS

MD 2013

Pino A., Randazzo C.L., Ricciardi L., Romano C., Comito D., Arena E., Saitta S., Caggia C. effect of probiotic *Lactobacillus reuteri* on gastrointestinal microbiota modulation and on clinical symptoms in patients with SNAS allergy.

Randazzo C.L., Solieri L., Pino A, Pitino I., Caggia C. integrated approach on 16S rDNA PCR-DGGE and hsp60 qPCR assay for monitoring dynamics of a L. rhamnosus probiotic strain in table olive fermentation.

PhD WORKSHOP 2014

Lactobacillus rhamnosus: a versatile probiotic species for food and human application

MD 2015

CARPINO S., RANDAZZO C.L., DE ANGELIS M., PINO A., RUSSO N., RAPISARDA T., BELVEDERE G., CAGGIA C. Tina biofilms as source of microorganisms involved in volatile organic compounds production of ragusano cheese.

RANDAZZO C.L., PINO A., Liotta L. (2), DE ANGELIS M., DE NARDO F., CHIOFALO V., TODARO A., GUGLIELMINO S., MAZZAGLIA A., CAGGIA C. Microbiological, physico-chemical and sensorial characterization of artisanal nicastrese goat cheese.

SOMED 2015

Pino A., Bartolo E., Caggia C., Ciancia A., Randazzo C.L. effect of Lactoferrin on vaginal microbiota of women with bacterial vaginosis. BEN GUSTAFFSON AWARD

ACKNOWLEDGMENT

I would like to thank to my PhD promoter, Professor Randazzo Cinzia Lucia, for supporting me during these past three years. Cinzia is someone you will instantly love and never forget once you meet her. She is the funniest advisor and one of the smartest people I know. I hope that I could be as lively, enthusiastic, and energetic as Cinzia and to someday be able to become a researcher as well she is. She has been supportive and has given me the freedom to pursue various projects without objection and also she provided insightful discussions about the research. I am also very grateful to my co-promoter Cinzia Caggia for her scientific advice and knowledge and many insightful discussions and suggestions. She was for me a key resource for getting my questions answered and for suggest me that I have to believe in my abilities. They were and remain my best role models for a scientist, mentor, and teacher. I also would like to thank Professors Willem de Vos and Erwin Zoetendal for the opportunity to spend 9 months of my PhD in the *moleco group* at Wageningen University. Also I thank Koenraad Van Hoorde for helping me to conclude the latest analysis. Some special words of gratitude go to my friends and Nunzia who have always been a major source of support when things would get a bit discouraging. I want to thank Emanuele, my parents and my sister Annalisa for their great support and encouragement and for simply being always there for me. Emanuele has been a true and great supporter and has unconditionally loved me during my good and bad times.