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Skin microbiota of first cousins affected by psoriasis and atopic dermatitis

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Introduction

The aim of this study was to investigate if there were any differences in the skin microbiota composition between three first cousins: 1 affected by psoriasis, 1 suffering of atopic dermatitis, and one who represented the healthy control.

Main Results

Significant differences between the skin microbiota of psoriatic individual and healthy and AD subjects were observed. The psoriatic subject showed a decrease in *Firmicutes* abundance and an increase in *Proteobacteria* abundance. Moreover, an increase in *Streptococcaceae*, *Rhodobacteraceae*, *Campylobacteraceae* and *Moraxellaceae* has been observed in psoriatic subject, if compared with AD individual and control. Finally, AD individual showed a larger abundance of *S. aureus* than psoriatic and healthy subjects. Moreover, the microbiota composition of non-lesional skin samples belonging to AD and psoriatic individuals was very similar to the bacterial composition of skin sample belonging to the healthy control.

Breakthrough

To reduce the variability between subjects, strict inclusion and exclusion criteria were adopted. Nonetheless, our results showed the need to deepen the analysis level to better highlight differences in the bacterial composition between individuals belonging to the study population.

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RESEARCH

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Skin microbiota of first cousins affected by psoriasis and atopic dermatitis

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Abstract

Background: Psoriasis and atopic dermatitis (AD) are chronic inflammatory skin diseases, which negatively influence the quality of life. In the last years, several evidences highlighted the pivotal role of skin bacteria in worsening the symptomatology of AD and psoriasis. In the present study we evaluated the skin microbiota composition in accurately selected subjects affected by (AD) and psoriasis.

Methods: Three first cousins were chosen for the study according to strict selection of criteria. One subject was affected by moderate AD, one had psoriasis and the last one was included as healthy control. Two lesional skin samples and two non-lesional skin samples (for AD and psoriatic subjects) from an area of 2 cm^2 behind the left ear were withdrawn by mean of a curette. For the healthy control, two skin samples from an area of 2 cm^2 behind the left ear were withdrawn by mean of a curette. DNA was extracted and sequencing was completed on the Ion Torrent PGM platform. Culturing of *Staphylococcus aureus* from skin samples was also performed.

Results: The psoriatic subject showed a decrease in *Firmicutes* abundance and an increase in *Proteobacteria* abundance. Moreover, an increase in *Streptococcaceae*, *Rhodobacteraceae*, *Campylobacteraceae* and *Moraxellaceae* has been observed in psoriatic subject, if compared with AD individual and control. Finally, AD individual showed a larger abundance of *S. aureus* than psoriatic and healthy subjects. Moreover, the microbiota composition of non-lesional skin samples belonging to AD and psoriatic individuals was very similar to the bacterial composition of skin sample belonging to the healthy control.

Conclusion: Significant differences between the skin microbiota of psoriatic individual and healthy and AD subjects were observed.

Keywords: Psoriasis, Atopic dermatitis, Metagenomics, Skin microbiota

Background

The largest organ of human body is the skin, which plays a pivotal role in protecting the host from pathogenic infections and penetration of harmful agents [1]. Before birth, the skin is completely sterile but after birth it is colonized by environmental microbes that are in homeostasis with the host [2, 3]. Moreover, after a vaginal delivery, fecal and vaginal microbes belonging to the mother's bacterial microflora also colonize the skin of infants. The microbial community living on the human skin is called skin microbiota, and it is constituted by over 100 distinct species of bacteria [4]. Generally, we find four dominant phyla of bacteria colonizing the skin: *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes* [5]. Several evidences exist about the pivotal role of bacteria in the development and persistence of atopic dermatitis (AD), a chronic itchy, inflammatory skin condition, very common in childhood. AD is considered to be a multifactorial disease, in which environmental and genetic factors contribute to its pathogenesis [1]. The incidence of AD has increased significantly in the last decades worldwide,

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leading the scientific community to hypothesize that the change in the lifestyle and nutrition may be involved in the development of aforementioned disease [5–7]. In the majority of individuals affected by AD, *Staphylococcus aureus* was found on the skin lesions. Furthermore, also the skin commensal *Staphylococcus epidermidis* has been observed to be increased with clinical disease activity, while bacteria belonging to the genera *Streptococcus*, *Propionibacterium* and *Corynebacterium* were increased only after a pharmacological therapy [8]. The skin microbiota dysbiosis may lead to a lack of immune system stimulation, together with an imbalance of type 1 T helper cells (T_H1) and type 2 cells (T_H2) activity, which might be involved in worsening of AD symptoms [9].

Psoriasis, instead, is a common inflammatory disease affecting 2–5 % of the population in industrialized countries. The disease is characterized by cutaneous inflammation and keratinocyte hyperproliferation, and it is often linked to severe complications, such as psoriatic arthritis [1]. Psoriasis has been hypothesized to result from a lack of immune tolerance to the skin microbiota in genetically predisposed individuals [10]. The aforementioned skin disease forms lesions on body often associated with beta-hemolytic streptococcal infection, where streptococcal superantigen leads T cell stimulation and expansion in the skin [11]. Several evidences highlighted the association between psoriasis and skin microbiota dysbiosis, underlying the relative abundance of *Corynebacterium*, *Propionibacterium*, *Staphylococcus* and *Streptococcus* in psoriasis plaques [12].

To date, the specific role of the skin microbiota in psoriasis and AD is still unknown, and it is unclear if the changes observed in the skin bacterial composition of individuals affected by psoriasis and AD are a cause or a consequence of alteration of the skin barrier, following the pathogenesis of aforementioned skin diseases. Certainly, the skin microbiota interacts with the host organism by producing several metabolites, which modulate cutaneous pro- and anti-inflammatory responses [13]. The current study used a next generation sequencing (NGS) approach to determine if some differences in the skin microbiota composition occur between individuals affected by psoriasis and AD, compared with a healthy control.

Methods

Study population

Three male first cousins aged 50 ± 3 years were chosen for the present study, according to specific and strict characteristics shared by all of them (Table 1). All subjects followed a diet rich in bifidogenic factors that promoted the growth of beneficial bacteria, such as bifidobacteria, and poor in allergenic foods that could worsening the symptomatology of psoriasis and atopic dermatitis. In particular, food containing vitamin A (peppers, carrots, spinach, basil, pumpkin), vitamin C (orange, lemon, and kiwi), folic acid (legumes, cereals, lettuce, asparagus), zinc (figs, sunflower seeds, potatoes) and omega-3 (fish) were allowed. In contrast, strawberries are often associated to allergies, in particular to atopic dermatitis, while tomato may lead to bowel dysfunction and it is not recommended for atopic subjects. Therefore, these foods were excluded from the diet of subjects enrolled in the study. Moreover, shellfish, cheese, fatty foods and red meat were not recommended (Table 1).

The selection criteria were chosen in agreement with dermatologists of the Clinical Dermatology Unit of IRCCS Galeazzi Orthopaedic Institute in Milan, Italy, where the study was conducted. One subject was affected by moderate AD, one had psoriasis and the last one was included as healthy control. The inclusion criteria for atopic dermatitis were moderate AD according to Hanifin and Rajka [14], with predominant rough and fissured skin as well as pruritus for at least 2 months. The inclusion criteria for psoriasis, instead, were the presence of psoriatic erythematous patches and the evaluation of psoriasis area and severity index (PASI) score, a tool used for the measurement of psoriasis severity. In particular, the PASI score of psoriatic subject enrolled in the study was 20.0, while the SCORAD for the AD subject was 32.46, underlying the presence of a moderate psoriasis and a moderate AD, respectively. Furthermore, subjects affected by psoriasis and AD had no concomitant diseases and both healthy and psoriatic individual had no any history of atopic dermatitis in childhood. The primary exclusion factors were the presence of chronic dermatosis such as seborrheic dermatitis, contact dermatitis, nummular eczema, ichthyosis, an immunodeficiency or any other immunological disorder, scabies, cutaneous fungal infection, HIV-associated skin disorders, malignant diseases, T-cell lymphoma, Letterer-Siwe disease, progressive systemic diseases, serious internal diseases (e.g., serious decompensated diseases of the heart, liver, and/or kidneys, or diabetes mellitus). The study was conducted according to ICH guidelines for Good Clinical Practice. All procedures followed were in accordance with the Declaration of Helsinki of 1975, as revised in 2000 and 2008. The study was approved by the Ethic Committee and Scientific Direction of the IRCCS Galeazzi Orthopaedic Institute. Subjects enrolled in the study provided verbal informed consent, which was carefully recorded in the study worksheets, to participate in the present study. Two lesional skin samples from a damaged area of 2 cm^2 behind the left ear were withdrawn by mean of a curette from AD and psoriatic subjects. Moreover, two non-lesional skin samples were taken from the

Table 1 Subjects' selection criteria

Subjects' characteristics	
Sex	Male
Age	50–53 years old
Subjects relationship	All individuals were first cousins
Diet	A Mediterranean diet was followed for 1 month before the day sampling. Pollen- and allergen- associated food, such as apple, hazelnut, celery, strawberries, shellfish and read meat were excluded from diet. Furthermore, milk intake was not recommended. Foods and beverages allowed were: bread, potatoes, vegetables, fresh fruit, meat and meat products, fish and fish products, eggs, edible fat, coffee, tea and soft drinks
Lifestyle	All individuals lived in the same neighborhood. No sport or daily exercises were practiced during the study period. No travel or excursion were carried out for at least 1 month before the day of sampling
Occupation	All subjects had a sedentary office work
Sexual activity	No sexual activity for 2 weeks before the day of sampling
Clothing	All individuals used only cotton clothes for all the study period
Pharmacological therapy	No antibiotic therapy was administered for at least 1 month before the day of sampling and they were not subjected to any kind of pharmacological therapy
Probiotic therapy	No probiotic therapy was administered for at least 1 month before the day of sampling
Personal care	All subjects used Cetaphil, a free-preservatives soap, once a day for 1 month before the day of sampling. Cetaphil is an oil-in-water petrolatum-based cream used to treat dry skin and often recommended for the management of AD. Moreover, no skin perfume or cream was used during the study
Others	No allergy to food, dust, pollen, grasses and drugs was present The subjects enrolled in the study shared no genetic diseases All subjects had not pets and/or contacts with any kind of animals All individuals were nonsmokers and not subjected to passive smoking The day of sampling took place the same day for all subjects enrolled in the study. The study was conducted during the winter season, in order to avoid excessive sweating

same area from AD and psoriatic individuals and used as internal control. For the healthy control, instead, two skin samples from an area of 2 cm^2 behind the left ear were withdrawn by mean of a curette. Samples were placed in sterile Petri dishes (one dish for each subject's sample) and stored at 4 °C until analysis. Of the two samples, one was used for metagenomics, and the other for cultural analysis. None of the individuals enrolled in the study was currently receiving specific therapy for psoriasis and AD (i.e. methotrexate, tacrolimus or pimecrolimus). No additional drugs (i.e. corticosteroids, anti-histamines) were used by subjects enrolled in the study. In particular, during the month before the day of sampling, the psoriatic individual was subjected to specific clinical investigations, such as screening for latent tuberculosis and rheumatoid arthritis, to determine the appropriate systemic therapy to be administered after the sampling. During this period, psoriatic individual could use lanolin, a skin protector, if necessary. The study was carried out after obtaining informed consent from all subjects, and in line with the guidelines for experimental studies on humans applicable within our Institute.

DNA extraction

Total DNA was extracted from skin samples using the Genomic DNA Mini Kit (Tissue) following the manufacturer's instructions (Geneaid, Italy). The protocol included an initial mechanical disruption step with a micropestle, followed by an enzymatic lysis incubation for 30 min at 60 °C.

16S rRNA gene amplification

Partial 16S rRNA gene sequences were amplified from extracted DNA using the 16S Metagenomics Kit (Life Technologies, Italy) that is designed for rapid analysis of polybacterial samples using Ion Torrent sequencing technology. The kit includes two primer sets that selectively amplify the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. The PCR conditions used were 10 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 20 s at 72 °C, followed by 10 min at 72 °C. Amplification was carried out by using a SimpliAmp thermal cycler (Life Technologies, Italy). The integrity of the PCR amplicons was analyzed by electrophoresis on 2 % agarose gel.

Ion torrent PGM sequencing of 16S rRNA gene-based amplicons

The PCR products derived from amplification of specific 16S rRNA gene hypervariable regions were purified by a purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter

Genomics, Germany) in order to remove primer dimers. From the concentration and the average size of each amplicon, the amount of DNA fragments per microliter was calculated and libraries created by using the Ion Plus fragment Library kit (Life Technologies, Italy). Barcodes were also added to each sample, using the Ion Xpress Barcode Adapters 1–16 kit (Life Technologies, Italy). Emulsion PCR was carried out using the Ion OneTouch TM 400 Template Kit (Life Technologies, Italy). Sequencing of the amplicon libraries was carried out on a 318 chip using the Ion Torrent Personal Genome Machine (PGM) system and employing the Ion PGM Hi-Q kit (Life Technologies, Italy) according to the supplier's instructions. After sequencing, the individual sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were also automatically trimmed. 16 rRNA sequences were then analyzed by Ion Reporter Software, which comprises a suite of bioinformatics tools that streamline and simplify analysis of semiconductor-based sequencing data. The 16S rRNA workflow module in Ion Reporter Software was able to classify individual reads combining a Basic Local Alignment Search Tool (BLAST) alignment to the curated Greengenes database, which contains more than 400,000 records, with a BLAST alignment to the premium curated MicroSEQ ID database, a high-quality library of full-length 16S rRNA sequences. The final output of Ion Reporter Software was the identification of microorganisms and their abundance in the sample.

Quantification of *Staphylococcus aureus* by means of cultivable method

Skin samples were transferred to 1.5 ml tubes and one ml of normal sterile solution (NaCl 9 g/l) was added. After homogenization to a homogeneous solution, samples were serially diluted in saline and appropriate dilutions were plated onto Mannitol Salt Agar (MSA), a selective medium for the growth of *Staphylococcus* spp, and incubated in aerobiosis for 48 h at 37 °C. All colonies of different morphology were identified according to: growth on selective medium, Gram staining, colony and cell morphology and the catalase and oxidase tests. The identification of *S. aureus* was performed by mean of RAPID Staph assay (Thermofisher, Italy). Finally, the percentage of occurrence of *S. aureus* in skin samples was calculated as follow:% *S. aureus* = [mean of CFU/cm^2 (*S. aureus*)]/[mean of log10 CFU/cm^2 (total staphylococci)] × 100.

Statistics

Differences in *S. aureus* counts between the three individuals were evaluated by means of Student t test.

Results

Metagenomics analysis of skin microbiota in AD and psoriatic individuals and in healthy control

Figures 1, 2 and 3 represent the comparison between the cutaneous microbiota of damaged skin from AD and psoriatic individuals and the skin of healthy control. Comparing lesional skin samples from AD and psoriatic subjects to the skin sample of healthy control, the most prevalent phyla detected in all subjects included *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. Subject affected by psoriasis showed a decrease in *Firmicutes* abundance and an increase in *Proteobacteria* abundance, if compared with AD patient and healthy individual (Fig. 1). A prevalence of *Proteobacteria* and *Bacteroidetes* over other phyla has been observed in psoriatic individual (Fig. 1). At family level, subject affected by psoriasis showed an increase in *Streptococcaceae*, *Rhodobacteraceae*, *Campylobacteraceae*, and *Moraxellaceae* if compared with control and AD patient (Fig. 2). However, even if psoriatic subject had a larger amount of *Rhodobacteriaceae*, a minor diversity in the family composition has been detected (Fig. 3). Indeed, in this subject the most abundant genus was *Paracoccus* (i.e. >90 % of total *Rhodobacteraceae* total reads) while in healthy and atopic individuals also *Rhodobacter* and *Haematobacter* were found in skin microbiota (Fig. 3). Furthermore, psoriatic patient showed a decrease in *Staphylococcaceae* and *Propionibacteriaceae* abundance if compared with AD and healthy individual (Fig. 2). In particular, the total skin microbiota of psoriatic individual showed a decrease in *Propionibacterium acnes* abundance (i.e. <2 % of total reads), while in AD and healthy subject *P. acnes* was more abundant (i.e. >10 % of total reads). *Lactobacillaceae* were the population that contributed in the minor proportion to the overall skin microbiota (i.e. <0.5 % of total reads). Interestingly, no differences between the skin microbiota composition of AD patient and healthy control has been observed (Figs. 1, 2). Comparing non-lesional skin samples from AD and psoriatic subjects to the skin sample of healthy control, we did not observed any differences in the skin microbiota composition; indeed, bacterial phyla (Fig. 4a) and family (Fig. 4b) composition in AD and psoriatic individuals was very similar to that observed in healthy subject. Furthermore, in non-lesional skin samples of AD and psoriatic individuals we observed a similar composition at genus level among the *Rhodobacteraceae* family, if compared with the healthy control (Fig. 4c).

Quantification of *S. aureus* in skin samples

Patient affected by AD showed the larger abundance of *S. aureus* (Fig. 5a), with a frequency of 73 % of *S. aureus* on the total staphylococci load found on skin sample. By

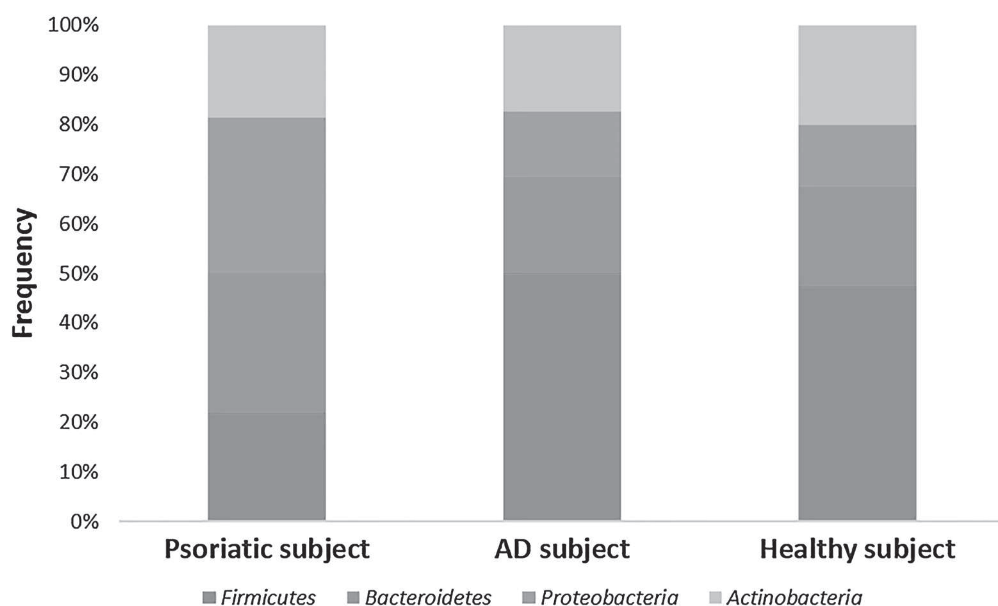

Fig. 1 Relative abundances of bacterial phyla on lesional skin samples of psoriatic and AD individuals and in the skin of healthy control

contrast, psoriatic individual had only a 2 % of *S. aureus* on his skin, while healthy control showed a frequency of 18 % (Fig. 5a). Moreover, no differences in *S. aureus* frequency were observed between non-lesional skin samples of AD and psoriatic subjects and healthy control (Fig. 5b).

Discussion

This is the first study in which the skin microbiota of AD and psoriatic selected individuals subjected to the same lifestyle and environment factors has been compared. Indeed, we evaluated the skin microbiota of much selected subjects affected by psoriasis and AD, compared with the microbiota of a healthy related control. In particular, we analyzed samples from the area behind the left ear, evaluating the microbiota associated to this specific anatomical district. One of the main factor that seem to be involved in the development of allergic diseases is the mode of delivery; indeed, cesarean section has been observed to be associated with a moderately risk of allergic rhinitis, asthma and hospitalization for asthma [15]. One hypothesis concerns the composition of the gut microbiota, which is established early in childhood. Vaginal delivery leads to the first colonization of infant gut with maternal vaginal and fecal bacteria, while cesarean babies are deprived of this natural exposure and present a different gut microbiota [15]. The first steps of infant gut colonization play a pivotal role in normal tolerance induction, as well as in the development and homeostasis of the immune system. Therefore, cesarean delivery may lead to an increased susceptibility to atopic conditions. Interestingly, all subjects enrolled in the present study were born by vaginal delivery and no correlations with the development of AD and psoriasis can be suggested. However, significant differences have been detected in the abundance of several bacterial family in subjects enrolled in the study. *Propionibacteriaceae*, indeed, showed a different distribution among the three groups analyzed, as AD and healthy individuals had a larger abundance of aforementioned bacterial family and its major human species *Propionibacterium acnes*, if compared with the subject affected by psoriasis. *P. acnes* are dominant microorganisms in normal skin [16–19], and a decrease in the number of these microbes could be a reflection of disordered ecological niches that become inhospitable to these microorganisms, and probably play a pivotal role in the pathogenesis of psoriasis or could be involved in the disease worsening [17]. *P. acnes* could have a beneficial and protective role on the human organism, and in particular, at skin level, by mean of its immunomodulatory action in signaling human cells [18, 19]. It has been hypothesized that *P. acnes* could protect the human skin from the action of several pathogenic bacteria, and its decrease could lead to a reduction of the protective skin barrier [17]. However, to date it is not clear if the decrease observed in *P. acnes* abundance is a consequence of the inflammatory state of psoriasis, or if this reduction is involved in the pathogenesis of the aforementioned disease.

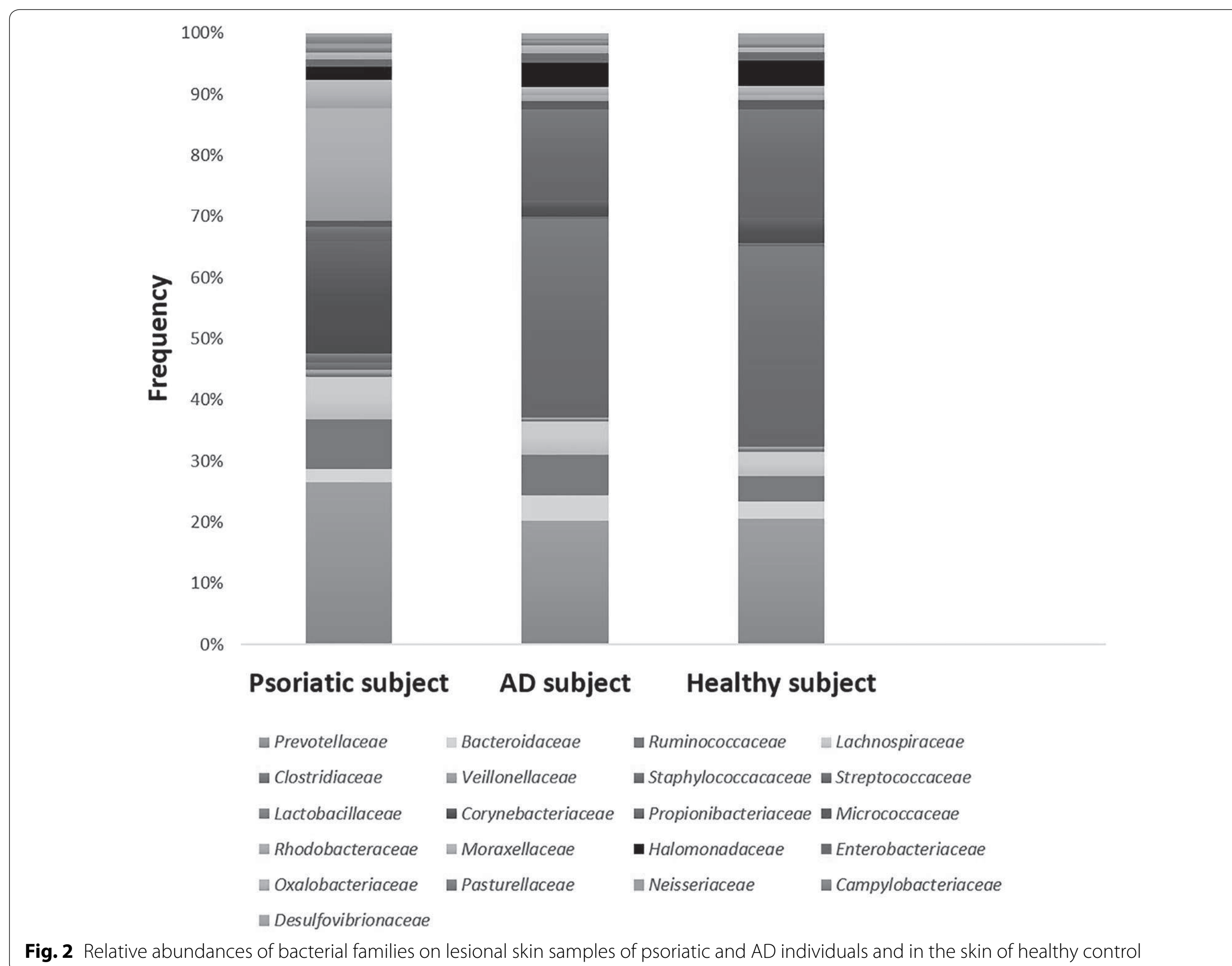

Fig. 2 Relative abundances of bacterial families on lesional skin samples of psoriatic and AD individuals and in the skin of healthy control

An increase in *Streptococcaceae* has been observed in psoriatic subject, confirming data of previous works, in which the presence of *Streptococcus* spp was linked to the pathogenesis of psoriasis [20, 21]. *Streptococcaceae* are highly relevant among the environmental factors that are involved in the development of psoriasis [21]. Different mechanisms, such as molecular mimicry, superantigens and the ability of streptococci for intracellular uptake and persistence in skin cells, may be involved in the pathogenesis of the disease. Furthermore, the skin microbiota associated to the subject with psoriasis showed a larger abundance of *Rhodobacteraceae*, in comparison to that of healthy and AD individual. Interestingly, a minor bacterial diversity has been observed in psoriatic sample, as *Paracoccus* was the predominant bacterial family detected. At the contrary, *Rhodobacter* and *Haematobacter* represented a significant part of the skin microbiota of AD and healthy subjects. *Rhodobacter* spp are able to produce a molecule, named lycogen, structurally similar to lycopene, which can acts as anti-inflammatory agent and as inhibitor of melanogenesis. This molecule is able to prevent the down-regulation of procollagen I and inhibit elevated production of NFκB, a transcription factor involved in cellular stress, which were elicited by UV-light exposure [22–24]. The absence of *Rhodobacteraceae* among the skin microbiota observed in psoriatic individual could be linked to the reduction of the physiological skin barrier integrity that is involved in the symptomatology and etiopathology of psoriasis.

Finally, our results did not underline a difference between the microbiota composition of AD individual and the healthy control, except for the higher frequency of occurrence of *S. aureus* in AD subject, even if a high abundance of *Staphylococcaceae* has been detected in both groups of individuals. Several evidences exist about the mainly role of *S. aureus* in the pathogenesis of AD [25–27]. In particular, the skin of AD subjects has been observed to be more frequently colonized by *S. aureus* if compared to that of healthy control. This microorganism is able to increase the skin inflammation by mean

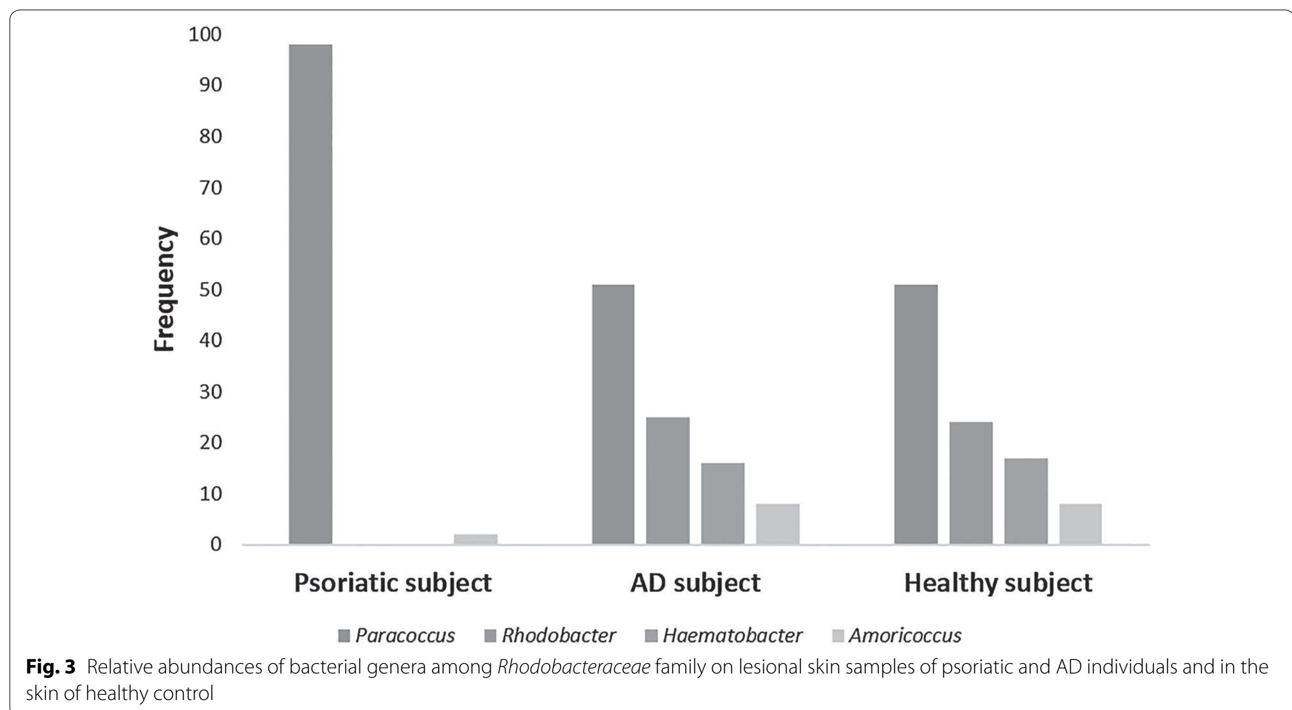

Fig. 3 Relative abundances of bacterial genera among *Rhodobacteraceae* family on lesional skin samples of psoriatic and AD individuals and in the skin of healthy control

of specific toxins which act as superantigens and, as a consequence, they may induce monocytes and lymphocytes activation, increasing also the production of several pro-inflammatory cytokines [28, 29]. Interestingly, as the severity of AD lesions increased, also the load of *S. aureus* has been observed to become higher. Probably, the high abundance of *S. aureus* we detected in AD sample contributed to worsening the skin barrier damages, leading to the characteristic skin lesions that affected AD subject enrolled in the present study. Moreover, the pivotal role of *S. aureus* in AD was underlined by the low frequency of *S. aureus* in non-lesional skin sample of AD individual, compared to the lesional skin sample of the same subject. Interestingly, the microbiota composition of non-lesional skin belonging to AD and psoriatic individuals was very similar to the bacterial composition of sample from the healthy control. These findings suggested that the cutaneous dysbiosis we observed in psoriatic and AD subjects was directly linked to the skin damages that characterize psoriasis and AD. Moreover, bacteria belonging to *Parococcus* genera, which have been observed to be increased in psoriasis skin sample, seem to be involved in the formation of skin pustules and they may have a direct role in the maintenance and worsening of psoriatic skin lesions [30]. Cutaneous bacteria have already been observed to be directly involved in the pathogenesis of several skin diseases, in particular in psoriasis and AD, where patients show a significant difference between the microbiota composition of lesional skin and non-lesional skin [31]. *S. aureus*, for example, is thought to play a pivotal role in the pathogenesis of these diseases, being involved not only in the pathology onset, but also in its progression, leading to an exacerbation of inflammatory response [31]. Moreover, differences in bacterial composition often observed in different skin sites may be directly involved in the different severity of cutaneous lesions on specific skin areas.

Although, several contradictory data about the specific role of cutaneous microbiota exist due to different sampling techniques. Some studies analyze the microbiota composition using skin swabs, while others investigate the bacterial composition from the complete epidermis and dermis, leading to different results [31]. To date, there are no enough information about the specific role of the skin microbiota in cutaneous diseases, as it is not clear if the bacterial dysbiosis often associated to these dyseases is the leading cause or a consequence of the pathological status. Interestingly, several evidences underlined that also intestinal bacteria seem to be directly involved in the onset and in the maintenance of allergic diseases, suggesting the possibility of preventing or treating AD and psoriasis by influencing the intestinal microbiota. The gut dysbiosis, associated to disruption of intestinal barrier function, may lead to a significant increase in local and systemic inflammation that is often associated to allergic diseases [32, 33]. AD patients, in particular, showed several changes in the gut microbiota composition, as bifidobacteria were significantly lower in comparison to

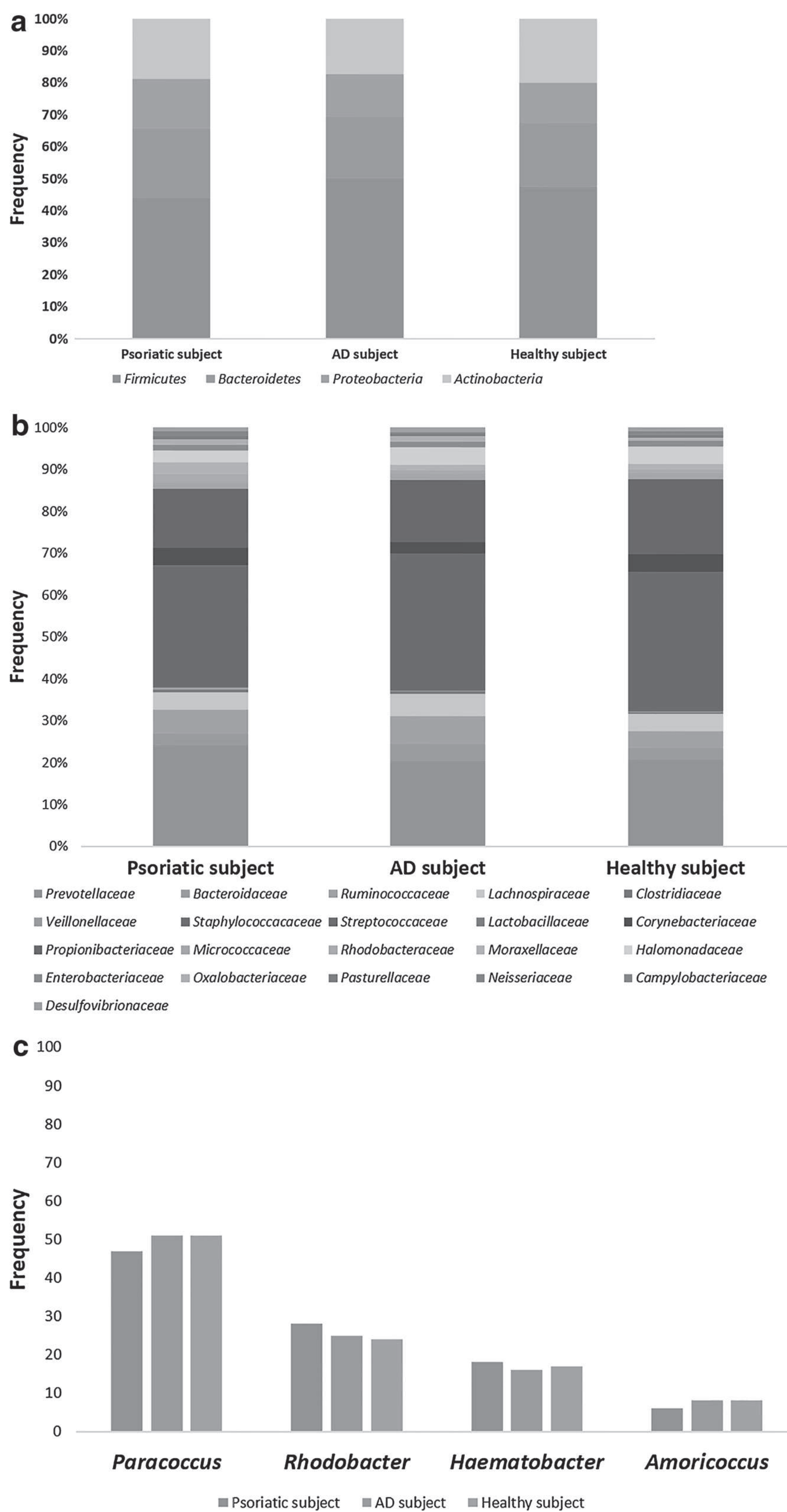

Fig. 4 Comparison between non-lesional skin samples of AD, psoriatic and healthy subjects. Comparison between bacterial phyla composition (**a**); comparison between bacterial family composition (**b**); comparison between bacterial genera composition among *Rhodobacteraceae* family (**c**)

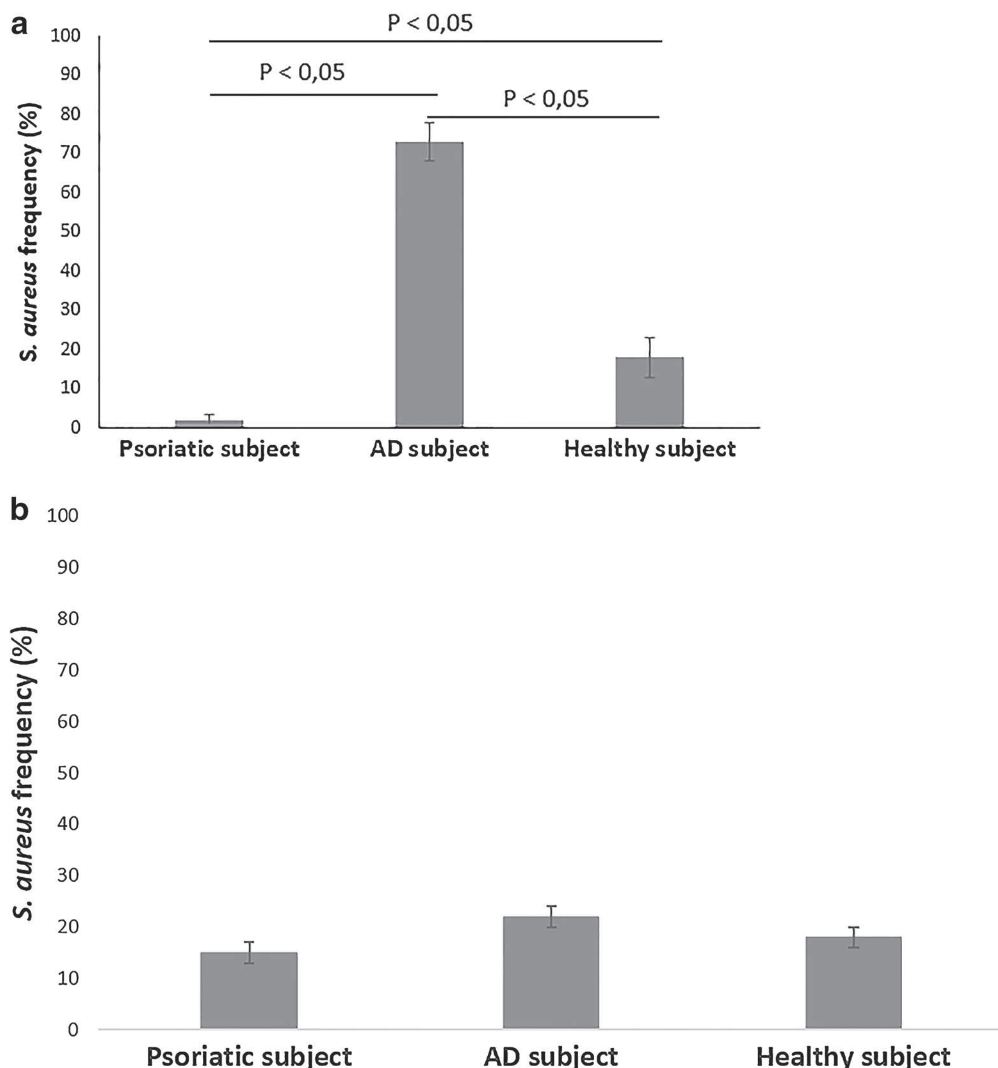

Fig. 5 Frequency of *S. aureus* recovery on the lesional skin samples of psoriatic and AD individuals and on the skin of healthy control (**a**); frequency of *S. aureus* recovery on the non-lesional skin samples of psoriatic, AD and healthy individuals (**b**). Differences were considered statistically significant at p value <0.05

healthy subjects, while the number of staphylococci was significantly higher in AD individuals compared with controls. Consequently, a decrease of anti-inflammatory molecules due to the reduction of bifidobacteria and a parallel increase in pro-inflammatory molecules due to the proliferation of staphylococci in the intestinal environment, may lead to the worsening of inflammation status and symptomatology of allergic diseases [32].

Conclusions

In conclusion, significant differences between the skin microbiota of psoriatic individual and healthy and AD subjects were observed. The majority of studies on AD- and psoriasis-associated skin microbiota focused their attention on the microbial population belonging to shoulder, finger, arm, forearm, elbow, abdomen, knee and leg, while we analyzed the area behind the left ear, highlighting how the skin area analyzed also could influence the microbiota composition in psoriasis and AD.

The forearm, for example, has been observed to be colonized mainly by microorganisms belonging to *Proteobacteria* and *Bacteroidetes* phyla, as well as the antecubital fossa that present also a high abundance of *Staphylococcaceae*. In popliteal fossa, instead, the most abundant microorganisms belong to the *Staphylococcaceae* family, while the inguinal crease is colonized mainly by *Corynebacterineae* [30]. Interestingly, the different skin sites present not only different kind of microorganisms,

but they differ also for evenness and richness of bacterial communities [34]. Consequently, when a dysbiosis of the skin microbiota occurs, the kind of bacteria located on various skin sites can differently influence the onset and the progression of cutaneous diseases.

However, one limit of the present study was the low number of subjects enrolled, but our purpose was to minimize all variables that could influence the skin microbiota composition, applying stringent criteria of patient selection. Moreover, another vial of the present study was the lack of information about the family history of AD and psoriasis in the maternal or paternal line. However, the choice to enroll these three correlated-individuals with similar lifestyle have been taken to reduce the study variables, as previous studies enrolled subjects with different lifestyles and geolocation that could influence the skin microbiota composition. Further analysis will be needed to examine greater number of lesions in order to evaluate the connection of skin microbiota composition with the progression of inflammatory diseases. Furthermore, also patients with severe AD might be involved in the study to verify the correlation between the skin microbiota, and in particular the presence of *S. aureus*, and the disease severity.

Authors' contributions

LD, GA, PP and OR designed the study. GA and PP collected clinical samples. MT and RDG processed clinical samples and performed metagenomics analysis. MT and RDG performed the statistical analysis. LD, GA, PP and OR interpreted data. LD and MT wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Manuscript 3

Ability of *Lactobacillus kefiri* LKF01 (DSM32079) to colonize the intestinal environment and modify the gut microbiota composition of healthy individuals

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Introduction

This study was aimed to investigate the ability of a new *L. kefiri* strain to reach the gut environment of healthy volunteers and modify their gut microbiota composition.

Main Results

L. kefiri LKF01 showed a strong ability to modulate the gut microbiota composition, leading to a significant reduction of several bacterial genera directly involved in the onset of pro-inflammatory response and gastrointestinal diseases. After one month of probiotic oral intake we observed a reduction of *Bilophila*, *Butyricicomonas*, *Flavonifractor*, *Oscillibacter* and *Prevotella*. Interestingly, after the end of probiotic administration *Bacteroides*, *Barnesiella*, *Butyricicomonas*, *Clostridium*, *Haemophilus*, *Oscillibacter*, *Salmonella*, *Streptococcus*, *Subdoligranolum* and *Veillonella* were significantly reduced if compared to baseline samples.

Breakthrough

In the present study we characterized the gut bacterial composition only at phyla and genera taxa for obtaining an overview of microbial changes that occurred after the probiotic oral intake. However, trying to offset the high interpersonal variability and obtain an integrated framework of information, we introduced a more detailed biodiversity analysis.

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Alimentary Tract

Ability of *Lactobacillus kefiri* LKF01 (DSM32079) to colonize the intestinal environment and modify the gut microbiota composition of healthy individuals

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ABSTRACT

Background: Probiotics have been observed to positively influence the host's health, but to date few data about the ability of probiotics to modify the gut microbiota composition exist.
Aims: To evaluate the ability of *Lactobacillus kefiri* LKF01 DSM32079 (LKEF) to colonize the intestinal environment of healthy subjects and modify the gut microbiota composition.
Methods: Twenty Italian healthy volunteers were randomized in pre-prandial and post-prandial groups. Changes in the gut microbiota composition were detected by using a Next Generation Sequencing technology (Ion Torrent Personal Genome Machine).
Results: L. kefiri was recovered in the feces of all volunteers after one month of probiotic administration, while it was detected only in three subjects belonging to the pre-prandial group and in two subjects belonging to the post-prandial group one month after the end of probiotic consumption. After one month of probiotic oral intake we observed a reduction of *Bilophila*, *Butyricicomonas*, *Flavonifractor*, *Oscillibacter* and *Prevotella*. Interestingly, after the end of probiotic administration *Bacteroides*, *Barnesiella*, *Butyricicomonas*, *Clostridium*, *Haemophilus*, *Oscillibacter*, *Salmonella*, *Streptococcus*, *Subdoligranolum*, and *Veillonella* were significantly reduced if compared to baseline samples.
Conclusion: L. kefiri LKF01 showed a strong ability to modulate the gut microbiota composition, leading to a significant reduction of several bacterial genera directly involved in the onset of pro-inflammatory response and gastrointestinal diseases.

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1. Introduction

The human gut microbiota plays a crucial role in both the maintenance of gastrointestinal homeostasis and host's health. Changes in its microbial composition may lead to an imbalance between the abundance of beneficial and harmful microorganisms. This condition, defined as dysbiosis, may favor the host susceptibility to several ailments including metabolic disorders, such as obesity and diabetes, inflammatory bowel disease (IBD) and atopic diseases. In the last years, the use of probiotics has been proposed as a potential therapeutic approach to restore the healthy gut bacterial composition and intestinal functions [1]. For this reason, nowadays there is an increasing interest in studying new bacterial strains that can be used for probiotic formulation. According to the official definition of World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO), probiotics are "live microorganisms, which when consumed in adequate amounts, confer a healthy effect on the host" [2]. Lactic acid bacteria (LAB) are considered the main probiotic microorganisms [3] among which lactobacilli represent the most studied bacteria. Although the mechanisms by which probiotics exert their potential beneficial activity have not yet been fully clarified, the ability to modulate the host's gut microbiota composition may play a pivotal role in maintaining the intestinal homeostasis [1]. To date, few data about the positive role of probiotics in modulating the gut microbiota of

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healthy individuals exist. A recent review highlighted no effects of probiotics on the gut microbiota in terms of diversity and richness of the intestinal bacterial composition [4,5]. However, small sample size, low resolution-methods of assessing fecal microbiota composition, different susceptibility toward the probiotic, the specific probiotic strain used in the study and variation in posology and probiotic formulation are all factors which can strongly confuse the conclusions of this work. Nonetheless, several studies highlighted the promising features of probiotics. Indeed, Drago et al. underlined the ability of *Lactobacillus salivarius* LS01 to improve the clinical and immunological status of patients affected by moderate/severe atopic dermatitis. This specific strain was able not only to lead a significant increase in the T-helper (Th1) response in atopic subjects but also to positively modulate the intestinal microbiota composition [6,7]. Indeed, a significant reduction of staphylococci, which are often involved in the development and progression of atopic dermatitis by producing several toxins with superantigenic properties, has been observed after the probiotic oral intake [6]. Moreover, *L. salivarius* LS01 has been observed to induce an anti-inflammatory response in allergic asthmatics individuals, leading to a reduction of Interleukin-13 (IL13) and Interleukin-17 (IL17), two pro-inflammatory cytokines involved in the worsening of allergic diseases [8]. Interestingly, the combination of *L. salivarius* LS01 and *Bifidobacterium breve* BR03 was able to significantly increase the production of anti-inflammatory cytokines by Peripheral blood mononuclear cells (PBMC) of asthmatic subjects if compared with the activity of each single strain [8].

Recently, different studies identified a great amount of LAB in kefir grains, or complex microbial community embedded in an extracellular polysaccharide matrix [9,10]. *Lactobacillus kefiri* LKF01 DSM32079 (LKEF) is a *lactobacillus* strain isolated from kefir grains which has already been tested for its susceptibility to several antibiotics [11]. Authors showed that *L. kefiri* LKF01 is safe for human consumption since, although it harbours some antibiotic resistance, there is not risk of antibiotic resistance transferability to intestinal pathogenic microorganisms. Though there are no evidences about the immunomodulatory activity of *L. kefiri* LKF01, a recent study highlighted the potential anti-inflammatory properties of *L. kefiri* CIDCA 8348, as it was able to enhance the production of IL-10 and significantly downregulate the expression of pro-inflammatory mediators in Peyer Patches and mesenteric lymph nodes [12]. In addition, *L. kefiri* P-IF strain was observed to act as a natural adjuvant for dendritic cells (DC) activation, upregulating the expression of DC surface co-stimulatory and maturation markers [13]. Consequently, *L. kefiri* strains seem having a beneficial impact on the host's health and they may be used to improve several gut inflammatory disorders. However, to date there are no studies evaluating the specific impact of *L. kefiri* strains on the human intestinal microbiota.

The aim of the present study was to investigate the effects of *L. kefiri* LKF01 DSM32079 (LKEF) on the gut microbiota composition of twenty healthy subjects. In addition, we evaluated if the pre- and post-prandial oral intake of *L. kefiri* influenced its ability to reach and colonize the human gastrointestinal tract.

2. Materials and methods

2.1. L. kefiri *LKF01 origin*

The *lactobacillus* strain was isolated from Water Kefir grains by Probiotical S.p.A. (Novara, Italy) that provided evidences about its safety for human consumption and characterized its probiotic features. The strain was deposited as DSM32079. The probiotic formulation used in the present study was manufactured by Probiotical S.p.A. (Novara, Italy).

2.2. *Experimental design*

Twenty Italian healthy subjects, four male and sixteen females, participated in the study. They were all volunteers who were informed in detail about the aim of the study. Baseline characteristics for each subject are summarized in Table 1. All individuals personally delivered fecal samples to the Laboratory of Clinical Microbiology (University of Milan, Milan). The experimental protocol was approved by the Scientific Direction of IRCCS Galeazzi Orthopaedic Institute in the Current Research 2015. Exclusion criteria included antibiotic treatment within the previous 2 months or suffering from any acute or chronic cardiovascular, gastrointestinal or immunological conditions. Furthermore, probiotics, yogurt and kefir grains have been excluded from the diet during the study period. All participants followed a Mediterranean diet from one month before the beginning of the study. In particular, the diet was rich in pasta, rice, red and white meat, fish, cheese, eggs, vegetables, potatoes, cereals and fruits. Sodas and alcoholic drinks were excluded from the diet, except for red and white wine, one glass per meal. Fried food was not included in the diet. Volunteers were randomized in two groups. Ten subjects took five drops of a suspension containing 10^{10} colony-forming units (CFU) of *L. kefiri*

Table 1
Baseline characteristics of healthy subjects enrolled in the study.

Sample ID	Gender	Age	Weight (kg)	Height (m)	Waist circumference (cm)	Fat mass (%)	Body mass index (BMI)
1	Male	37	70,0	1,80	83	20	21,6
2	Female	35	65,0	1,65	89	15	23,9
3	Female	30	57,0	1,64	70	2	21,2
4	Female	42	60,0	1,68	80	10	21,3
5	Female	41	72,0	1,75	73	3	23,5
6	Female	38	70,0	1,83	90	10	20,9
7	Female	36	62,0	1,73	80	9	20,7
8	Female	42	73,0	1,72	85	12	24,7
9	Female	43	71,5	1,70	82	10	19,8
10	Female	39	64,0	1,80	80	9	19,8
11	Female	38	64,0	1,79	72	3	20,0
12	Male	43	63,0	1,73	75	19	21,0
13	Female	41	67,0	1,69	82	12	23,5
14	Male	35	64,0	1,77	80	9	20,4
15	Female	37	61,0	1,72	77	7	20,6
16	Female	40	60,0	1,62	75	0	22,9
17	Female	44	54,0	1,59	80	3	21,4
18	Male	36	60,0	1,80	75	21	18,5
19	Female	45	65,0	1,79	76	6	20,3
20	Female	40	60,0	1,65	90	2	22,0

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LKF01 DSM32079 (LKEF) 30 min before breakfast (pre-prandial administration), while ten subjects took 5 drops of the probiotic suspension 30 min after breakfast (post-prandial administration). The probiotic drops were diluted in water at room-temperature. During the month of *L. kefiri* oral intake, no problems of drops resuspension have been highlighted. Moreover, the product was tasteless and odorless, favoring the daily oral intake. Fecal samples were collected from each participant one week before the probiotic oral intake, after one month of probiotic administration and one month after the end of probiotic consumption. The study was conducted according to ICH guidelines for Good Clinical Practice. All procedures followed were in accordance with the Declaration of Helsinki of 1975, as revised in 2000 and 2008. One subject was excluded from the study following an antibiotic therapy, while 2 individuals were excluded from the data analysis as they did not strictly follow the diet.

2.3. DNA extraction

Total DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Italy).

2.4. Quantitative PCR

In order to assess the ability of *L. kefiri* LKF01 to colonize the human gut microbiota a quantitative species-specific real-time PCR was carried out using a Rotor Gene 3000 system (Diatech, Italy). For *L. kefiri* DNA amplification the following primers were used: LK1 (forward) 5′ CAACAATCAAAGGGGTTGTTG 3′ and LK2 (reverse) 5′ TCACTAGGAGTAATTGAACCA 3′ (Eurofin, Vimodrone, Italy). The two primers amplify a genomic region of 439 bp belonging to rpoA gene which encodes for the RNA polymerase alpha subunit.

The reaction conditions for DNA amplification were 95 °C for 10 min, 40 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and 72 °C for 10 min. The quantification protocol used to identify the abundance of fecal *L. kefiri* LKF01 was that published by Castillo et al. [14].

2.5. 16S rRNA gene amplification

Partial 16S rRNA gene sequences were amplified from extracted DNA using the 16S Metagenomics Kit (Life Technologies, Italy) that is designed for rapid analysis of polybacterial samples using Ion Torrent sequencing technology. The kit includes two primer sets that selectively amplify the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. The PCR conditions used were 10 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 20 s at 72 °C, followed by 7 min at 72 °C. Amplification was carried out by using a SimpliAmp thermal cycler (Life Technologies, Italy). The integrity of the PCR amplicons was analyzed by electrophoresis on 2% agarose gel.

2.6. Ion torrent PGM sequencing of 16S rRNA gene-based amplicons

The PCR products derived from amplification of specific 16S rRNA gene hypervariable regions were purified by a purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics, Germany) in order to remove primer dimers. DNA concentration of the amplified sequence library was estimated through the Qubit system (Life Technologies, Italy). From the concentration and the average size of each amplicon, the amount of DNA fragments per microliter was calculated and libraries created by using the Ion Plus Fragment Library kit (Life Technologies, Italy). Barcodes were also added to each sample, using the Ion Xpress Barcode Adapters 1–16 kit (Life Technologies, Italy). Emulsion PCR was carried out using the Ion OneTouch TM 400 Template Kit (Life Technologies, Italy) according to the manufacturer's instructions. Sequencing of the amplicon libraries was carried out on a 318 chip using the Ion Torrent Personal Genome Machine (PGM) system and employing the Ion PGM Hi-Q kit (Life Technologies, Italy) according to the supplier's instructions. After sequencing, the individual sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were also automatically trimmed. 16 rRNA sequences were then analyzed by Ion Reporter Software, which comprises a suite of bioinformatics tools that streamline and simplify analysis of semiconductor-based sequencing data. The 16S rRNA workflow module in Ion Reporter Software was able to classify individual reads combining a Basic Local Alignment Search Tool (BLAST) alignment to the curated Greengenes database, which contains more than 400,000 records, with a BLAST alignment to the premium curated MicroSEQ ID database, a high-quality library of full-length 16S rRNA sequences. In the first step, reads were aligned to the MicroSEQ ID library with any unaligned reads subject to a second alignment to the Greengenes database to achieve rapid and exhaustive bacterial identification. The final output of Ion Reporter Software was the identification and abundance of microorganisms at phyla, class, family and genus level.

2.7. Statistical analysis

The biodiversity index (Shannon, Simpson and Chao) and statistical analyses were carried out using the R Software V.3.3.1, for Windows. Non-parametric Kruskal–Wallis and Mann–Whitney tests were used to find significant differences in α diversity and microbial taxa. Adjustment for multiple testing was evaluated with Dunn's post-hoc test p-values below 0.05 were considered statistically significant.

3. Results

In all subjects no side effects were observed following the oral intake of *L. kefiri* LKF01.

3.1. Colonization ability of L. kefiri LKF01

After one month of *L. kefiri* LKF01 administration, the *lactobacillus* strain was recovered in the feces of all subjects enrolled in the study with a bacterial load of 10^5–10^6 CFU/g of feces, after both pre-prandial and post-prandial administration. One month after the end of probiotic treatment, *L. kefiri* was recovered only in three subjects belonging to the pre-prandial group and in two subjects belonging to the post-prandial group with a bacterial load of 10^3 CFU/g of feces (Fig. 1, panel A and B, respectively).

3.2. Effects of L. kefiri LKF01 on the gut microbiota biodiversity

To evaluate the bacterial diversity in the gut microbiota before and after the *L. kefiri* LKF01 consumption, as well as after the end of probiotic administration we calculated, for each time point, the Shannon, Simpson and Chao index reported in Fig. 2. No significant differences in the biodiversity have been observed after *L.* kefiri LKF01 oral intake. A slight decrease of Chao index after the end of probiotic administration was observed but it was not significant.

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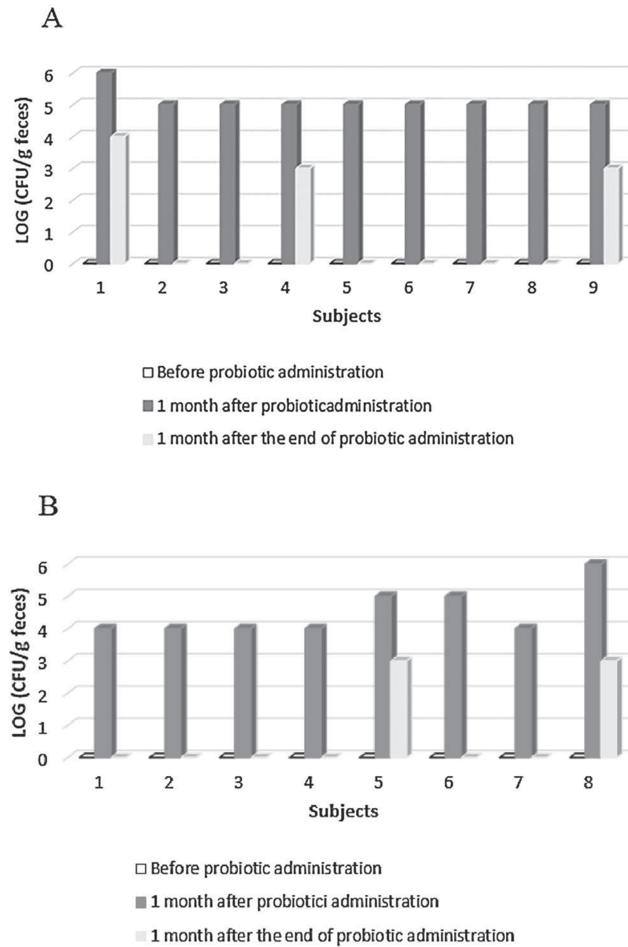

Fig. 1. Bar graphs represent the abundance of *L. kefiri* LKF01, expressed as CFU/g feces, detected in each subject belonging to the pre-prandial group (A) and post-prandial group (B).

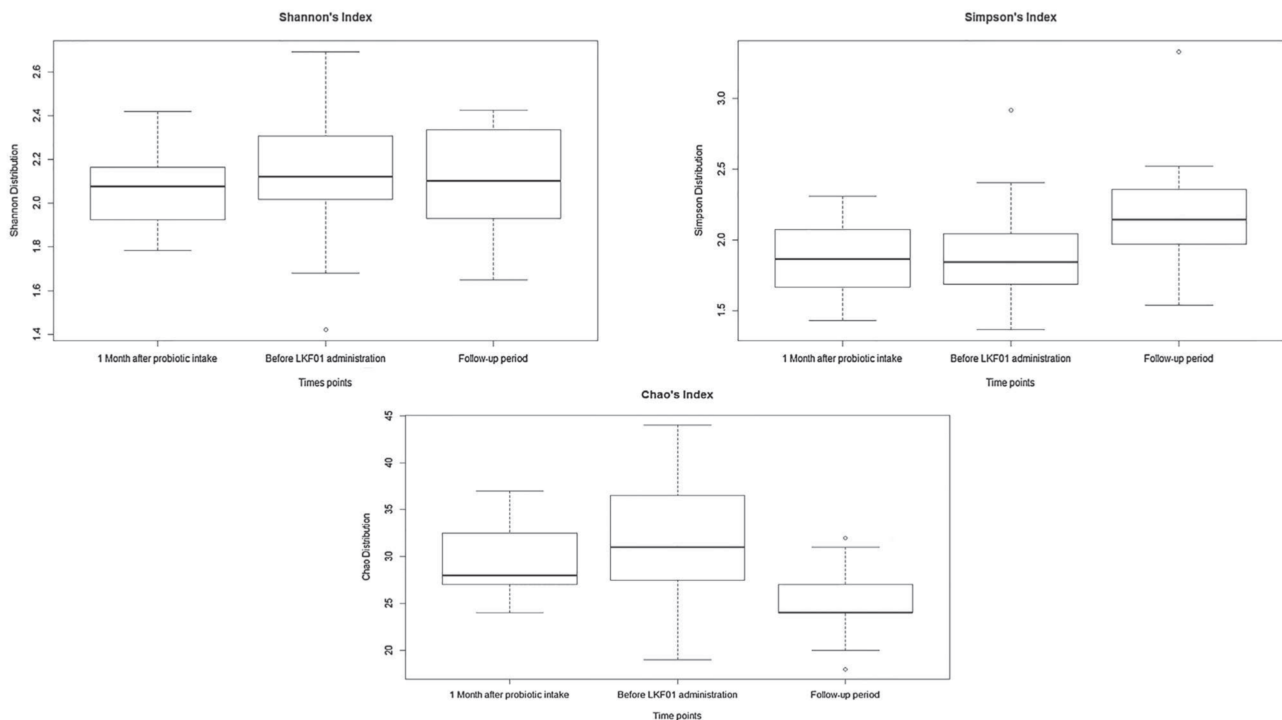

Fig. 2. Shannon's index, Simpsons's index and Chao's index before probiotic oral intake, one month after probiotic administration and one month after the end or *L. kefiri* consumption. Mann–Whitney tests were performed for each pair wise comparison and Kruskal–Wallis test across all three groups.

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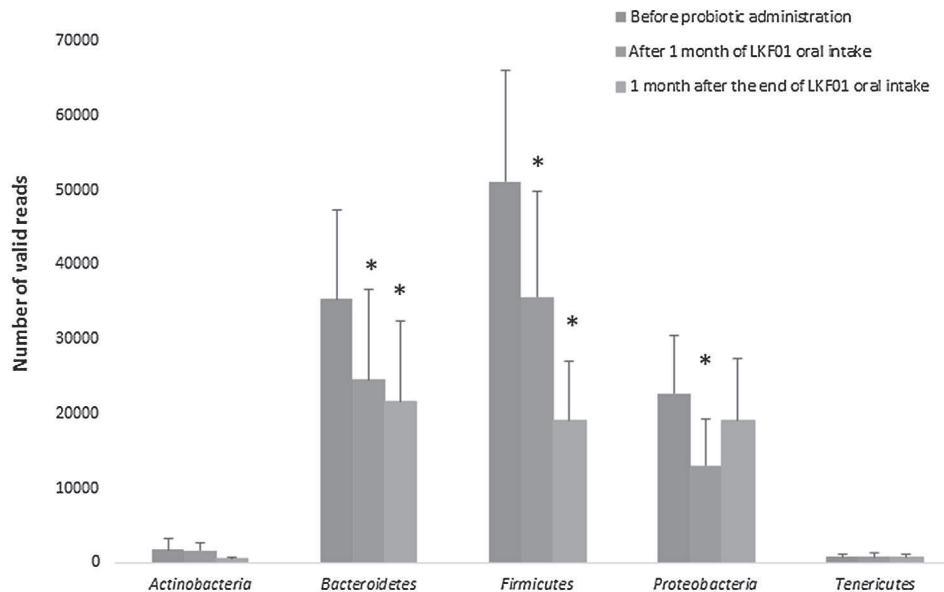

Fig. 3. Distribution of the main bacterial phyla detected in fecal samples before LKF01 oral intake, after one month of probiotic administration and one month after the end of probiotic oral intake. Significant reduction is indicated by an asterisk (p value <0.05).

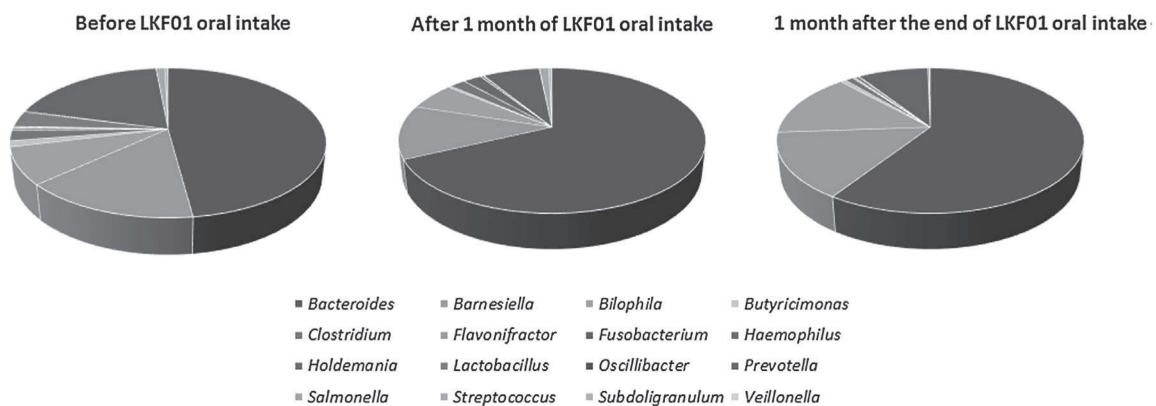

Fig. 4. Distribution of the main bacterial genera detected in fecal samples before LKF01 oral intake, after one month of probiotic administration and one month after the end of probiotic oral intake.

3.3. Bacterial changes at phylum level in the gut microbiota composition after the L. kefiri *LKF01 consumption and one month after the end of probiotic oral intake*

The distribution of the main bacterial phyla characterizing the gut microbiota of individuals enrolled in the study is shown in Fig. 3. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were subjected to a significant reduction (37%, 30% and 43%, respectively) after one month of LKF01 oral intake (Fig. 3). Furthermore, one month after the end of probiotic administration *Bacteroidetes* maintained the same reduction rate (about 30%), while *Firmicutes* abundance was further decreased (reduction of 63% if compared to samples collected before probiotic administration).

3.4. Bacterial changes at genus level in the gut microbiota composition after the L. kefiri *LKF01 consumption and one month after the end of probiotic oral intake*

Fig. 4 shows the distribution of the main bacterial genera found in the present study before and after the probiotic administration.

Lactobacillus population was increased of 93% after the oral intake of *L. kefiri* (data not shown), but this increase was not maintained in the follow-up period as the lactobacilli load returned to the baseline value (data not shown). Moreover, significant differences were detected in the gut microbiota composition after one month of *L. kefiri* LKF01 consumption, as samples showed a decreased abundance of *Bilophila* spp., *Butyricicomonas* spp., *Flavonifractor* spp., *Oscillibacter* spp. and *Prevotella* spp. if compared to samples collected at the beginning of the experimental time course ($p < 0.05$) (Fig. 5A). Interestingly, one month after the end of probiotic oral intake we observed a greater impact of *L. kefiri* on the gut microbiota composition, as several bacterial changes were detected in the follow-up period but not immediately after the probiotic administration. Indeed, a significant decrease in *Bacteroides* spp., *Barnesiella* spp., *Butyricicomonas* spp., *Clostridium* spp., *Haemophilus* spp., *Oscillibacter* spp., *Salmonella* spp., *Streptococcus* spp., *Subdoligranolum* spp., and *Veillonella* spp. has been detected (Fig. 5B). Furthermore, *L. kefiri* LK01 administration led no changes in the abundance of *Fecalibacterium prausnitzii* (data not shown) up to one month after the end of probiotic intake.

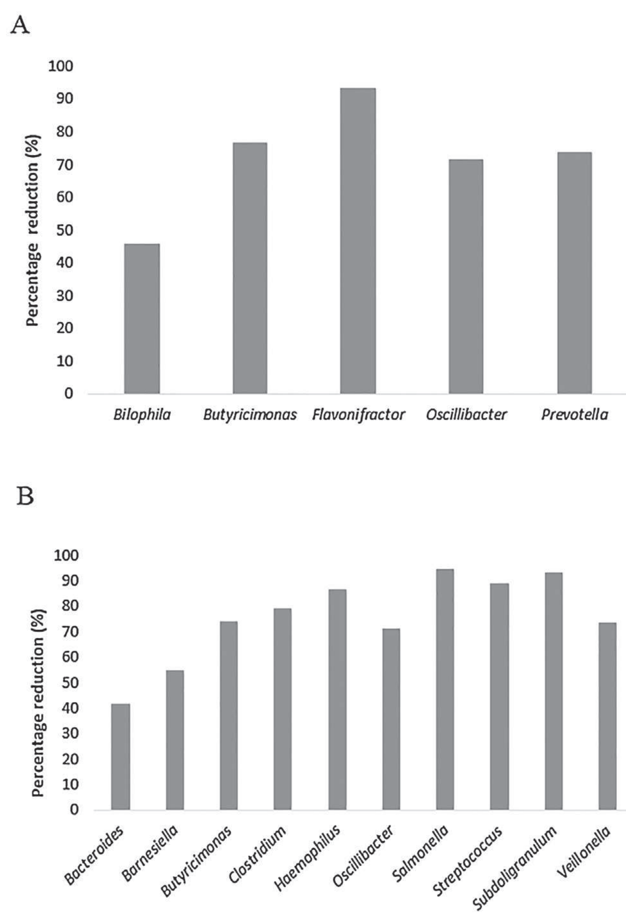

Fig. 5. Significant percentage reduction of several intestinal bacterial genera after 1 month of *L. kefiri* administration (A) and 1 month after the end of probiotic treatment (B) ($p < 0.05$).

4. Discussion

In the last years, several studies supported the hypothesis that probiotic microorganisms may have a potential role in conferring beneficial effects to the host's health [1,3,15,16].

The majority of probiotic bacteria are able to adhere to intestinal cells and colonize the gut environment, exerting different beneficial effects, many of which are mediated mainly by the number of viable cells achieving the gut. To date, the literature suggests that a continued probiotic consumption is needed to obtain a sustained impact on the human health [17]. Our data underline the high colonization capability of *L. kefiri* LKF01, which is not only able to survive in the gastric environment, but also to persist and be viable in the human gut. Indeed, the colonization ability is fundamental for a probiotic microorganism to exert its beneficial effect on the host's organism, as it becomes able to counteract intestinal pathogenic microorganisms and stimulate the immune system.

In this study, we did not perform a strain-specific PCR, but *L. kefiri* was not recovered before the probiotic oral intake and, moreover, all volunteers did not use probiotics or kefir grains during the study period. Consequently, not being *L. kefiri* an intestinal microorganism, it is likely presuming that its recovery in fecal samples was directly due to the probiotic administration. Although *L. kefiri* induced several changes in the gut microbiota composition, it did not lead to a reduction of microbial biodiversity. This is a noteworthy result since the scientific community agrees that the loss of the gut microbiota complexity may play a pivotal role in the onset or progression of numerous diseases such as obesity and irritable bowel syndrome (IBS). Consequently, the administration of *L. kefiri* would not seem having a negative impact on the general health status of the host. Contrariwise, *L. kefiri* LKF01 seems able to exert a beneficial effect on the bacterial ecology of the gastrointestinal tract, as many significant positive changes in the intestinal bacteria composition have been highlighted.

The probiotic consumption led to a significant reduction of *Firmicutes* and *Bacteroidetes*, the two major phyla characterizing the gut microbiota, and *Proteobacteria*, which is involved in the maintenance of a balanced gut microbial community [18]. In particular, an increased prevalence of *Proteobacteria* is often associated to a high risk of developing intestinal dysbiosis and gastrointestinal diseases [18]. Consequently, the reduction of this bacterial phylum after the probiotic oral intake highlights the potential protective role of *L. kefiri* LKF01 toward the intestinal health.

Furthermore, the higher amount of lactobacilli which was observed after one month of probiotic oral intake, seems to be positively correlated with the decrease of some bacterial genera which can act as pathogens, even if there is no information about the specific role of *Bilophila*, *Butyricicomonas*, *Flavonifractor* and *Oscillibacter* that were reduced immediately after the probiotic oral intake. Within the bacterial taxa modulated by *L. kefiri*, *Bacteroides* spp. and *Prevotella* spp. have also been detected. Many species belonging to these bacterial genera are normally commensals of the human gut microbiota. However, these microorganisms can also be responsible for infections with significant morbidity and mortality. Various species of *Bacteroides* group were found in the majority of clinical infections as responsible for abscess formation. *Bacteroides distasonis*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* are often involved in different pathologic processes including intra-abdominal infections, colon cancer and periodontal diseases, exhibiting an increased resistance to many antibiotics [19]. Similarly, some *Prevotella* species can act as potential opportunistic pathogens, such as *Prevotella intermedia* and *Prevotella copri* that are able to penetrate tissues and cause different infections, especially after surgery or trauma [20]. Interestingly, both *Prevotella* and *Bacteroides* are bindweed in the stimulation of pro-inflammatory cytokines, such as Interleukin 6 (IL6), which is often involved in the worsening of several human diseases. Consequently, the significant reduction of these bacterial genera observed in the present study can be considered as a positive probiotic mark which contributes maintaining stable the host's health [21,22]. Surprisingly, the impact of *L. kefiri* on the gut microbiota was stronger one month after the end of the probiotic administration, as more bacterial genera have been observed to be less abundant if compared to the beginning of the experimental time course. The *L. kefiri* oral intake led to a significant reduction of *Haemophilus*, *Clostridium*, *Salmonella*, *Veillonella* and *Streptococcus* which, as well as *Bacteroides* and *Prevotell*a, can act as negative modulators of the host's immune system. Indeed, these microorganisms induce a profound inflammatory response by intoxicating intestinal epithelial cells causing pro-inflammatory cytokines release and promoting the onset of IBS and several other enteric diseases [23–27]. In particular, *Veillonella* and *Streptococcus* can metabolically interact in the intestinal ecosystem combining their immunomodulatory activities, leading to a greater pro-inflammatory response [27]. In this context, *L. kefiri* LKF01 seems to have a strong anti-inflammatory activity which is not exerted only by a direct stimulation of the intestinal immune system, but also by the modulation of specific microorganisms with a pro-inflammatory asset.

Moreover, we did not observe any changes in the abundance of *F. prausnitzii*, a commensal bacterium which synthesizes butyrate and other short-chain fatty acids through the fermentation of dietary fibers. Several studies showed that *F. prausnitzii* is able to promote an anti-inflammatory response, having a protective role in IBD

development and progression [28,29] and for this reason it is considered a beneficial microorganism which contributes improving the human's health. As a consequence, the compatibility between *F. prausnitzii* and *L. kefiri* LK01 is promising to ensure the effectiveness of both microorganisms in the intestinal environment. Finally, we have no information about the role of *Barnesiella*, *Butyricimonas*, *Oscillibacter* and *Subdoligranolum* and the meaning of their intestinal reduction is not still clear.

In conclusion, in this study we have shown that *L. kefiri* LKF01 has promising probiotic features which make it the best candidate for the development of a new probiotic product whose intake is not affected by the administration timing. Indeed, the ability to colonize the intestinal environment and positively modify the gut microbiota composition is a fundamental characteristic for a probiotic strain to promote and maintain the host's health.

Further studies are needed to better understand the specific mechanisms involved in the gut microbiota modulation and the real meaning of aforementioned changes observed after the probiotic oral intake.

Conflict of interest

None declared.

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Manuscript 4

Effect of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium longum* BB536 on the healthy gut microbiota composition at phyla and species level: a preliminary study

Submitted after revision in World journal of Gastroenterology (January 2017)

Introduction

The aim of this work was to evaluate the effect of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 on the gut microbiota composition of healthy volunteers. The aforementioned probiotic strains have already been well-characterized and demonstrated to have a positive impact on host's health.

Main Results

B. longum BB536 and *L. rhamnosus* HN001 showed the ability to modulate the gut microbiota composition, leading to a significant reduction of potential harmful bacteria and to an increase of beneficial ones. Immediately following 1 month of probiotic administration, the *B. longum* BB536 and *L. rhamnosus* HN001 load was found to have increased in the majority of subjects in both pre-prandial and post-prandial groups. This increase was still found 1 month after the end of probiotic oral intake in both groups, if compared to samples collected before probiotic consumption. At phyla level a significant decrease in *Firmicutes* abundance was detected immediately after 1-month of *B. longum* BB536 and *L. rhamnosus* HN001 oral intake. This reduction persisted up to 1 month after the end of probiotic oral intake together with a significant decrease of *Proteobacteria* abundance if compared to samples collected before probiotic administration.

Breakthrough

In this study, we applied the biodiversity analysis and evaluated microbial changes at phyla and species levels. Although this kind of characterization allowed us to obtain a sufficiently detailed view of intestinal microbiota composition, our results demonstrated a lack of information regarding the potential relationships between microorganisms.

1 Manuscript Type: **Original article**

2 **TITLE PAGE**

3 Effect of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium longum* BB536 on the healthy gut

4 microbiota composition at phyla and species level: a preliminary study

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6

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14 **Author contributions**: Lorenzo Drago led the conception of manuscript and contributed to draft it;

15 Marco Toscano and Roberta De Grandi performed experiments, analyzed data and prepared the

16 manuscript; Laura Stronati and Elena De Vecchi contributed to the acquisition of samples, analysis

17 and interpretation of data.

18 **Conflict-of-interest statement**: There is no conflict of interest.

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22

Abstract

AIM: To evaluate the ability of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium longum* BB536 to colonize the intestinal environment of healthy subjects and modify the gut microbiota composition.

METHODS: Twenty healthy Italian volunteers, eight males and twelve females, participated in the study. Ten subjects took a sachet containing 4 x 10^9 colony-forming units (CFU) of *Bifidobacterium longum* BB536 and 10^9 colony-forming units (CFU) of *Lactobacillus rhamnosus* HN001, 30 minutes before breakfast (pre-prandial administration), while ten subjects took a sachet of probiotic product 30 minutes after breakfast (post-prandial administration). The ability of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium longum* BB536 to colonize human gut microbiota was assessed by bean of quantitative real-time PCR, while changes in gut microbiota composition were detected by using Ion Torrent Personal Genome Machine.

RESULTS: Immediately after 1-month of probiotic administration, *B. longum* BB536 and *L. rhamnosus* HN001 load was increased in the majority of subjects in both pre-prandial and post-prandial groups. This increase was found also 1 month after the end of probiotic oral intake in both groups, if compared to samples collected before probiotic consumption. At phyla level a significant decrease in *Firmicutes* abundance was detected immediately after 1-month of *B. longum* BB536 and *L. rhamnosus* HN001 oral intake. This reduction persisted up to 1 month after the end of probiotic oral intake together with a significant decrease of *Proteobacteria* abundance if compared to samples collected before probiotic administration.

Whereas, at species level, a higher abundance of *Blautia product*, *Blautia wexlerae* and *Haemophilus ducrey* was observed, together with a reduction of *Holdemania filiformis, Escherichia vulneris, Gemmiger formicilis* and *Streptococcus sinensis* abundance. In addition, during follow-up period we observed a further reduction in *Escherichia vulneris* and *Gemmiger formicilis*, together with a decrease in *Roseburia faecis* and *Ruminococcus gnavus* abundance. Conversely, the abundance of *Akkermansia muciniphila* was increased if compared to samples collected at the beginning of the experimental time course

CONCLUSION:

B. longum BB536 and *L. rhamnosus* HN001 showed the ability to modulate the gut microbiota composition, leading to a significant reduction of potentially harmful bacteria and an increase of beneficial ones. Further studies are needed to better understand the specific

mechanisms involved in gut microbiota modulation.

Key words: Probiotics; Gut microbiota; *Bifidobacterium; Lactobacillus*; Human health.

Core tip

Several studies have described the potentially beneficial effects of many probiotic microorganisms belonging to *Lactobacillus* and *Bifidobacterium* genera. We evaluated the ability of *Bifidobacterium longum* BB536 *and Lactobacillus rhamnosus* HN001, two probiotic strains used in combination, to colonize the intestinal environment of healthy subjects and modify the gut microbiota composition. We did not observe a negative impact of probiotic on the general health status of the hosts. Contrariwise, the two bacterial strains seemed

69 able to exert a beneficial effect on the bacterial ecology of the gastrointestinal tract, as

70 many significant positive changes in gut microbiota composition have been highlighted.

71

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78 **Main text**

79 **INTRODUCTION**

80 Probiotics are defined as "non-pathogenic live micro-organisms that, when administered
81 in adequate amounts, confer a health benefit on the host" [1]. In the last years, their
82 numerous beneficial properties and positive impact on human health have deeply been
83 described [2]. Consequently, the global market for probiotics is growing due to the
84 increased demand of consumers who use these products to improve their health and even
85 to prevent some human illnesses such as allergic and gastrointestinal diseases, modulate
86 immune system and ensure the homeostasis of intestinal microbiota [3-5]. Nowadays,
87 hundreds of different bacterial strains are available in the global probiotic market and
88 consequently, the choice of the most suitable probiotic product becomes very difficult and
89 fragmented. For these reasons, safety and efficacy of probiotics are considered the main
90 criteria for using any microorganism in the formulation of probiotic products [6].
91 *Lactobacilli* and *Bifidobacteria* are the main microorganisms used as probiotics; indeed,
92 numerous species belonging to these genera have been reported as safe and effective in
93 improving the host's health [7]. Interestingly, modulation of intestinal microbiota
94 composition has been proposed as one of the main mechanisms of probiotic activity [8].
95 Several studies showed that the combination of specific bacterial strains belonging to
96 *Lactobacillus* and *Bifidobacterium* species can act in optimal synergy for restoring the
97 intestinal balance [9–11]. In 2015, Drago et al. (2015), for instance, highlighted the
98 immunomodulatory synergy of *L. salivarius* LS01 and *Bifidobacterium breve* BR03 which
99 combination led to an increased immunomodulatory activity if compared to the activity of
100 each single strain [12]. Also *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001
101 are two well-characterized probiotic strains often used in combination which ability to

102 survive the adverse gastrointestinal conditions and adhere to intestinal mucosa has
103 already been demonstrated in a previous study [13]. These probiotic strains possess strong
104 immunomodulatory activities, are able to improve human health, reduce eczema
105 prevalence in children and inhibit adhesion of gram-negative pathogens in intestinal
106 environment [14-22].
107 The aim of the present study was to investigate the effects of *B. longum* BB536 and *L.*
108 *rhamnosus* HN001 on the gut microbiota composition of healthy subjects after one month
109 of probiotic oral intake, also evaluating the potential impact of pre- and post-prandial
110 probiotic administration on the colonization ability of *Bifidobacterium longum* BB536 and
111 *Lactobacillus rhamnosus* HN001.
112

113 **MATERIALS AND METHODS**

114 **Experimental design**

115 Twenty healthy Italian subjects, eight males and twelve females, participated in the study.
116 They were all volunteers who were informed in detail about the aim of the study. Baseline
117 characteristics for each subject are summarized in Table 1. All individuals personally
118 delivered fecal samples to the Laboratory of Clinical Microbiology (University of Milan,
119 Milan). The experimental protocol was approved by the Scientific Direction of IRCCS
120 Galeazzi Orthopaedic Institute in the Current Research 2015.
121 Exclusion criteria included antibiotic treatment within the previous 2 months or suffering
122 from any acute or chronic cardiovascular, gastrointestinal or immunological conditions.
123 Furthermore, probiotics and yogurt have been excluded from the diet during the study
124 period. Volunteers were randomized in two groups. The probiotic product was provided
125 by Alfa Wassermann S.p.a. (Milan, Italy) that also supplied evidence about its safety for

126 human consumption. Ten subjects took a sachet containing 4 x 10^9 colony-forming units
127 (CFU) of *Bifidobacterium longum* BB536 and 10^9 colony-forming units (CFU) of *Lactobacillus*
128 *rhamnosus* HN001 30 minutes before breakfast (pre-prandial administration), while ten
129 subjects took a sachet of probiotic product 30 minutes after breakfast (post-prandial
130 administration). During the probiotic resuspension and before oral intake, some
131 volunteers described the formation of lumps inside the solution. Moreover, the product
132 was tasteless and odorless, favoring the daily oral intake. Fecal samples were collected
133 from each participant one week before the probiotic oral intake, after one month of
134 probiotic administration and one month after the end of probiotic consumption. Two
135 subjects were excluded from the study following an antibiotic therapy, while 2 individuals
136 were excluded from the data analysis as they did not strictly follow the diet.

137 **DNA extraction**

138 Total DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit
139 following the manufacturer's instructions (Qiagen, Italy).

140 **Quantitative PCR**

141 The ability of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 to colonize
142 the human gut microbiota was assessed by means of a quantitative real-time PCR carried
143 out using a Rotor Gene 3000 system (Diatech), in order to evaluate the potential increase of
144 the lactobacilli and bifidobacteria load following the probiotic oral intake. For *B. longum*
145 DNA amplification the following primers were used: *B. longum* (forward) 5′- TTC CAG
146 TTG ATC GCA TGG TC -3′ and *B. longum* (reverse) 5′- GGG AAG CCG TAT CTC TAC
147 GA -3′ (Eurofin, Vimodrone, Italy). The reaction conditions for DNA amplification were
148 94°C for 5 minutes, 35 cycles of 94°C for 20 seconds, 55°C for 20 seconds, 72°C for 20
149 seconds and 72°C for 5 minutes. Differently, for *L. rhamnosus* DNA amplification the PCR

150 reaction was performed using the following primers: *L. rhamn* (forward) 5′-

151 TGCATCTTGATTTAATTTTG-3′ and *L. rhamn (*reverse) 5′-

152 CCACTGCTGCCTCCCGTAGGAGT-3′ (Eurofin, Vimodrone, Italy). The amplification

153 profile was an initial step of 94 °C for 3 minutes, and then 30 cycles of 94 °C for 45 seconds,

154 55 °C for 45 seconds and 72 °C for 1 minute.

155 **16S gene sequencing**

156 DNA amplification and 16S gene sequencing were performed as previously described

157 (Drago *et al*., 2016).

158 **Statistical analysis**

159 The biodiversity index (Shannon, Simpson and Chao) and statistical analyses were carried

160 out using the R Software V.3.3.1, for Windows. Non-parametric Kruskal-Wallis and Mann-

161 Whitney tests were used to find significant differences in α diversity and microbial taxa.

162 Adjustment for multiple testing was evaluated with Dunn's post-hoc test. P-values below

163 0.05 were considered statistically significant.

164 **Biostatistics statement**

165 The statistical methods of this study were reviewed by

166 **RESULTS**

167 In all subjects no side effects were observed following the oral intake of probiotic product.

168 **Colonization ability of *Bifidobacterium longum* BB536 and *Lactobacillus***

169 ***rhamnosus* HN001**.

170 After one month of probiotic administration, an increase of *B. longum* load was

171 detected in 6 of 8 subjects belonging to the pre-prandial group (Figure 1A). This

172 increase was maintained 1 month after the end of the probiotic consumption. In 2

173 individuals (subjects 3 and 5) the *B. longum* load after the end of the treatment was

174 higher than that detected immediately after the probiotic oral intake (Figure 1A). In
175 the post-prandial group, however, we observed an increase of *B. longum* abundance
176 up to 1 month after the end of the probiotic in 4 subjects (Figure 1A). Only in one
177 individual (subject 13), 1 month after the end of the probiotic oral intake the *B.*
178 *longum* load was higher than both baseline samples and samples taken immediately
179 after the probiotic oral intake (Figure 1A). Moreover, after 1 month of probiotic
180 administration an increase of *L. rhamnosus* load was observed in 5 of 8 subjects
181 belonging to the pre-prandial group (Figure 1B). This increase was also found 1
182 month after the end of the probiotic, although *L. rhamnosus* abundance was slightly
183 lower than that detected immediately after the month of probiotic administration
184 (Figure 1B). In the post-prandial administration group, *L. rhamnosus* was increased in
185 4 subjects up to 1 month after the end of probiotic oral intake.

186 **Effects of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001**
187 **on gut microbiota biodiversity**

188 To evaluate the bacterial diversity and richness in the gut microbiota before and after
189 the intake of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 we
190 calculated, for each time point, Shannon's, Simpson's and Chao's indices reported in
191 Figure 2. Although no significant differences in gut microbiota biodiversity were
192 observed after *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 oral
193 intake, a slight reduction of Chao's index was detected one month after the end of
194 probiotic administration.

195 **Bacterial changes at phylum level in the gut microbiota composition after the probiotic**
196 **consumption and one month after the end of probiotic oral intake**

197 Distribution of main bacterial phyla characterizing the gut microbiota of individuals

enrolled in the study is shown in Figure 3. *Firmicutes* were subjected to a significant reduction (about 50%) after one month of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 oral intake (Figure 3). Moreover, one month after the end of probiotic administration, a further reduction of *Firmicutes* (about 20%) was observed, together with a significant decrease of *Proteobacteria* abundance (about 58%), if compared to samples collected before probiotic administration.

Bacterial changes at species level in gut microbiota composition after the probiotic consumption and one month after the end of probiotic oral intake

Significant differences at species level detected in gut microbiota are shown in Figure 4. Immediately after the probiotic administration, a significant increase of *Blautia product, Blautia wexlerae* and *Haemophilus ducrey* load was observed, together with a reduction of *Holdemania filiformis, Escherichia vulneris*, *Gemmiger formicilis* and *Streptococcus sinensis* abundance (Figure 4A).

Interestingly, the reduction of *Holdemania filiformis* remained stable up to one month after the end of probiotic intake ($p<0.05$) (Figure 4B).

In addition, during the follow-up period a further decrease in *Escherichia vulneris* and *Gemmiger formicilis* load, together with a reduction of *Roseburia faecis* and *Ruminococcus gnavus* abundance was observed (Figure 4B). Differently, an opposite trend was highlighted for *Akkermansia muciniphila* which abundance was increased if compared to samples collected at the beginning of the experimental time course ($p<0.05$) (Figure 4B).

DISCUSSION

To date, there is little evidence about the ability of probiotics to influence the gut microbiota composition and many factors can negatively influence conclusions drawn from different studies. The administration of different probiotic species or strains, the

222 duration of probiotic administration, the use of monostrain or multistrains products and
223 the presence of numerous variables related to the host's lifestyle are all elements which
224 can lead to different scientific conclusions [23].
225 In the present study, the timing of probiotic administration did not significantly influence
226 the colonization ability of *B. longum* BB536 and *L. rhamnosus* HN001. Indeed, both
227 probiotic strains were capable to colonize the intestinal environment independently of the
228 pre- or post-prandial oral intake. Furthermore, the two probiotic strains did not influence
229 the gut microbiota diversity and richness of healthy individuals, as no significant changes
230 in the Shannon, Simpson and Chao indices were detected. However, the Chao index was
231 slightly decreased one month after the end of probiotic oral intake, suggesting a certain
232 degree of uniformity between the gut microbiota of different subjects analyzed. These
233 results confirmed data of a previous work which highlighted no significant effects of
234 probiotics on the gut microbiota diversity and richness [23]. The lack of activity of certain
235 probiotic strains on the gut microbiota could be related to their inability to colonize the
236 intestinal environment. The ability to adhere to and colonize the gut, indeed, is a
237 fundamental feature for probiotic microorganisms to be effective on the host and is closely
238 species- and strain-dependent [24]. We assume that *B. longum* BB536 and *L. rhamnosus*
239 HN001 had a good colonization ability: an increase in *B. longum* and *L. rhamnosus* fecal
240 load was detected in the majority of subjects analyzed after the probiotic supplementation.
241 They also had an impact on the gut microbiota composition at phylum level; indeed, a
242 significant reduction of *Firmicutes* was detected after one month of probiotic oral intake.
243 This result may be of importance since a high abundance of *Firmicutes* has previously been
244 related to obesity, and with a reduction of *Bacteroidetes* [25], as obese individuals often show
245 an unbalanced ratio of *Firmicutes* and *Bacteroidetes* in their intestinal microbiota.

246 Researchers hypothesized that the *Firmicutes* phylum contains numerous bacterial species
247 with an increased ability to harvest energy from diet, leading to a large increase in total
248 body fat [25]. Interestingly, the further reduction of *Firmicutes* we observed after the end of
249 probiotic administration was concomitant to a significant reduction of *Proteobacteria*, a
250 bacterial phylum often involved in the onset and progression of gastrointestinal diseases
251 [25]. In particular, different microorganisms belonging to *Proteobacteria*, such as
252 *Campylobacter*, enterohepatic *Helicobacter* and *Escherichia coli* are often associated with the
253 pathogenesis of Inflammatory Bowel Disease (IBD), being able to negatively influence the
254 immune system and enhance intestinal inflammation [26]. Consequently, these changes can
255 be considered positive marks due to *B. longum* BB536 and *L. rhamnosus* HN001, which
256 seem to be acting as beneficial biomodulators of gut microbiota. Changes observed at
257 phylum level were then confirmed by analyzing the distribution of intestinal bacterial
258 species after probiotic oral intake; indeed, the *Escherichia vulneris*, *Gemmiger formicilis* and
259 *Ruminococcus gnavus* load was reduced after probiotic administration. These
260 microorganisms have numerous mechanisms, including secretion of toxins and
261 colonization factors, for inducing the imbalance of intestinal homeostasis and leading to
262 the the onset of gastrointestinal diseases [27-29]. In particular, *Ruminococcus gnavus* may
263 possess different pathogenic traits and virulence factors, as it was already observed being
264 involved in two cases of bacteremia associated with diverticular disease [29]. Similarly,
265 *Gemmiger formicilis*, together with *Ruminococcus lactaris* and *Enterococcus durans*, has been
266 observed to be significantly increased in individuals with Chron's disease and subjected to
267 recurrence of inflammatory lesions [30]. Probably, these bacteria can act as pro-
268 inflammatory modulators enhancing the inflammatory state and worsening
269 symptomatology associated to the disease. Interestingly, the anti-inflammatory effect *B.*

270 *longum* and *L. rhamnosus* strains was further highlighted by the greater abundance of
271 *Blautia producta* and *Blautia wexlerae* during the follow-up period if compared to samples
272 collected at the beginning of the study and after one month of probiotic administration.
273 *Blautia* spp, indeed, produce short-chain fatty acids (SCFA), which act as main fuel for
274 enterocytes, and anti-inflammatory compounds involved in the promotion of muscular
275 activity and epithelial cell proliferation and in the enhancement of blood through the
276 colonic vasculature [31, 32]. Moreover, several studies have already demonstrated the ability
277 of both *B. longum* BB536 and *L. rhamnosus* HN001 to decrease the severity of allergic
278 responses and positively stimulate a host's immune system [33, 34]. The latter, in particular,
279 could be mediated by both a direct interaction between probiotic strains and cells of the
280 immune system, and modulation of intestinal microorganisms able to influence pro- and
281 anti-inflammatory response. Considering this, the regulation of gut microbiota
282 composition by probiotic bacteria takes on an even more important role in maintaining a
283 host's health. Intestinal microbiota, indeed, participating in the regulation of the immune
284 system, is closely involved in the onset or manifestation of allergic diseases, such as atopic
285 dermatitis [35]. A dysbiotic microbiota can enhance intestinal and cutaneous pro-
286 inflammatory response by production of metabolites and toxins with a strong
287 inflammatory power [35]. Intestinal *Staphylococcus aureus*, for instance, which was observed
288 to be more abundant in patients affected by moderate/severe atopic dermatitis, is able to
289 produce a toxin with superantigenic properties that exacerbates atopic symptomatology
290 [36].

291 More interestingly, the beneficial impact that *B. longum* and *L. rhamnosus* strains had on
292 intestinal homeostasis is underlined by the significant increase of *Akkermansia municiphila*
293 detectable only after the end of probiotic intake. *A. municiphila* is closely related with

294 human health and it is inversely associated with body fat mass and glucose intolerance [37].
295 Moreover, this bacterium seems to be involved in the maintenance of intestinal barrier
296 functions and above all in prevention of intestinal inflammation, playing a pivotal role in
297 the host's overall health status [38]. Also, the persisting reduction of *Streptococcus sinensis*
298 one month after the probiotic supplementation can be considered a positive mark for
299 probiotic supplementation, as *S. sinensis* is a potential pathogenic microorganism which
300 could be directly involved in infective endocarditis [39]. Consequently, its decrease can
301 further underline the beneficial impact that *B. longum* BB536 and *L. rhamnosus* HN001
302 administration may have on gut microbiota of healthy individuals.
303 In conclusion, our preliminary data highlighted the probiotic activity exerted by a *B.*
304 *longum* BB536 and *L. rhamnosus* HN001 combination which influences the intestinal
305 environment. The two probiotic strains have been demonstrated to influence the gut
306 microbiota composition, even if all bacterial changes detected after probiotic intake have
307 not yet been well-characterized.
308 However, a reduction of potential harmful bacteria and an increase of beneficial ones may
309 constitute an important probiotic feature of *B. longum* BB536 and *L. rhamnosus* HN001. Of
310 course, we need further clinical and pre-clinical studies to demonstrate a clear application
311 of this probiotics combination in the clinical field.
312

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456

457 **ILLUSTRATIONS**

458

459 **Figure 1**

460 Colonization ability of *Bifidobacterium longum* BB536 (panel A) and *Lactobacillus*
461 *rhamnosus* HN001 (panel B). Bar graphs represent the abundance of each bacterial strain,
462 expressed as colony-forming unit per gram of feces (*CFU/g feces*), recovered from each
463 subject belonging to the pre-prandial group and post-prandial group.

464

465 **Figure 2**

466 The Shannon, Simpson and Chao's indeces before probiotic oral intake (T0), one month after
467 probiotic administration (T1) and one month after the end (T2) of *Lactobacillus rhamnosus* HN001
468 and *Bifidobacterium longum* BB536 consumption. Mann–Whitney tests were performed for each
469 pair wise comparison and the Kruskal–Wallis test across all three groups.

470

471 **Figure 3**

472 Distribution of the main bacterial phyla detected in fecal samples before *Lactobacillus rhamnosus*
473 HN001 and *Bifidobacterium longum* BB536 consumption, after one month of probiotic
474 administration and one month after the end of probiotic oral intake. Any significant reduction is
475 indicated by an asterisk (p value <0.05).

476

477 **Figure 4**

478 Significant changes in the gut bacterial species composition: (A) bacterial distribution after 1 month
479 of probiotic oral intake; (B) bacterial distribution one month after the end of probiotic (follow up) (p
480 value <0.05).

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486 **Table 1** Baseline characteristics of health volunteers

Subject	Gender	Age	Weight (Kg)	Height (m)	Body Mass Index (BMI)
Pre-prandial group					
1	Female	40	70,0	1,80	21.6
2	Female	32	65,0	1,65	23.9
3	Male	45	57,0	1,64	21.2
4	Male	42	73.0	1,68	25.9
5	Male	35	85.0	1,90	23.5
6	Male	30	82.0	1,83	24.5
7	Male	31	71.0	1,68	25.2
8	Male	43	82.0	1,82	24.8
Post-prandial group					
9	Male	28	71,0	1,70	24.6
10	Male	29	64,0	1,80	19.8
11	Male	33	81.0	1,79	25.3
12	Male	43	72.0	1,73	24.1
13	Male	41	81.0	1,79	25.3
14	Male	35	71.0	1,77	22.7
15	Female	35	61,0	1,72	20.6
16	Female	33	60,0	1,62	22.9

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Figure 1

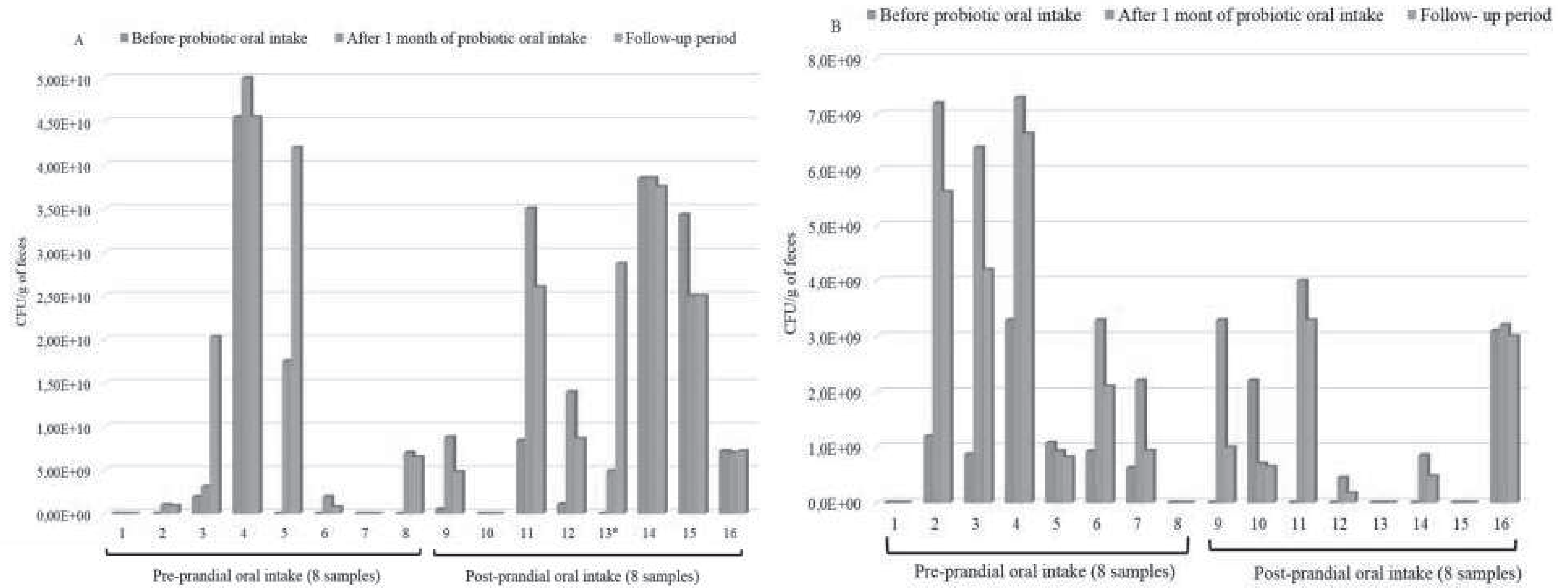


Figure 2

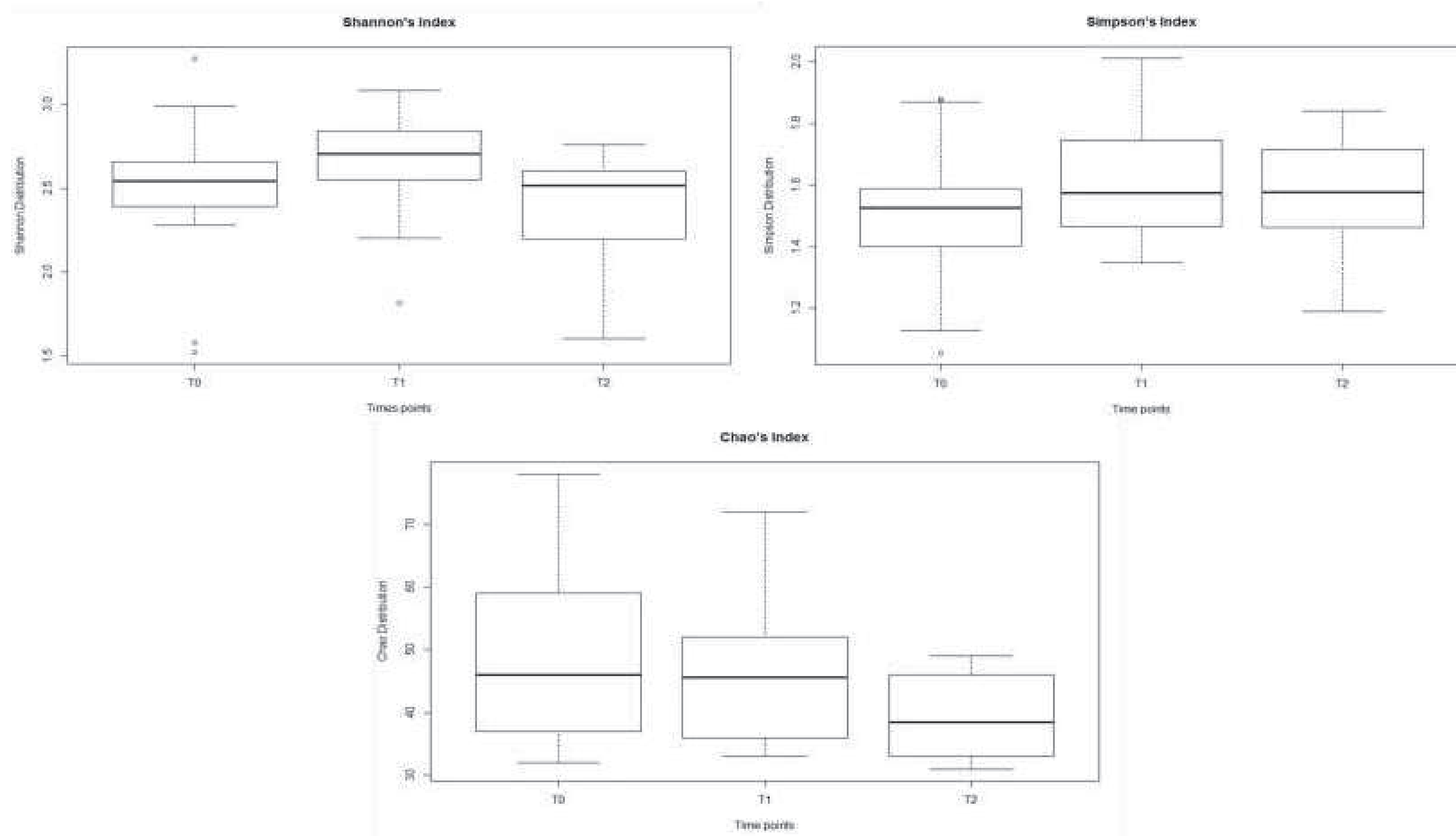


Figure 3

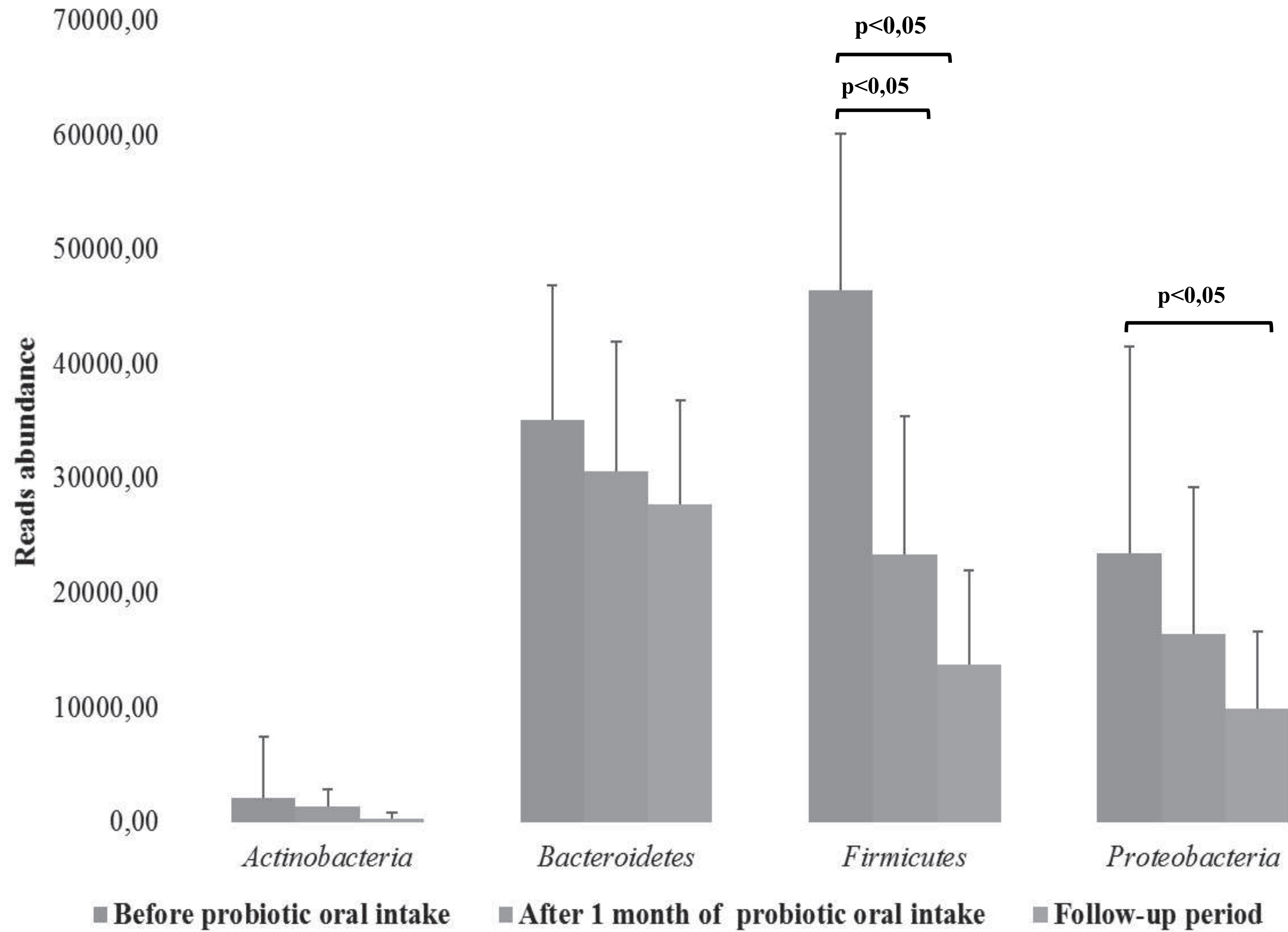


Figure 4

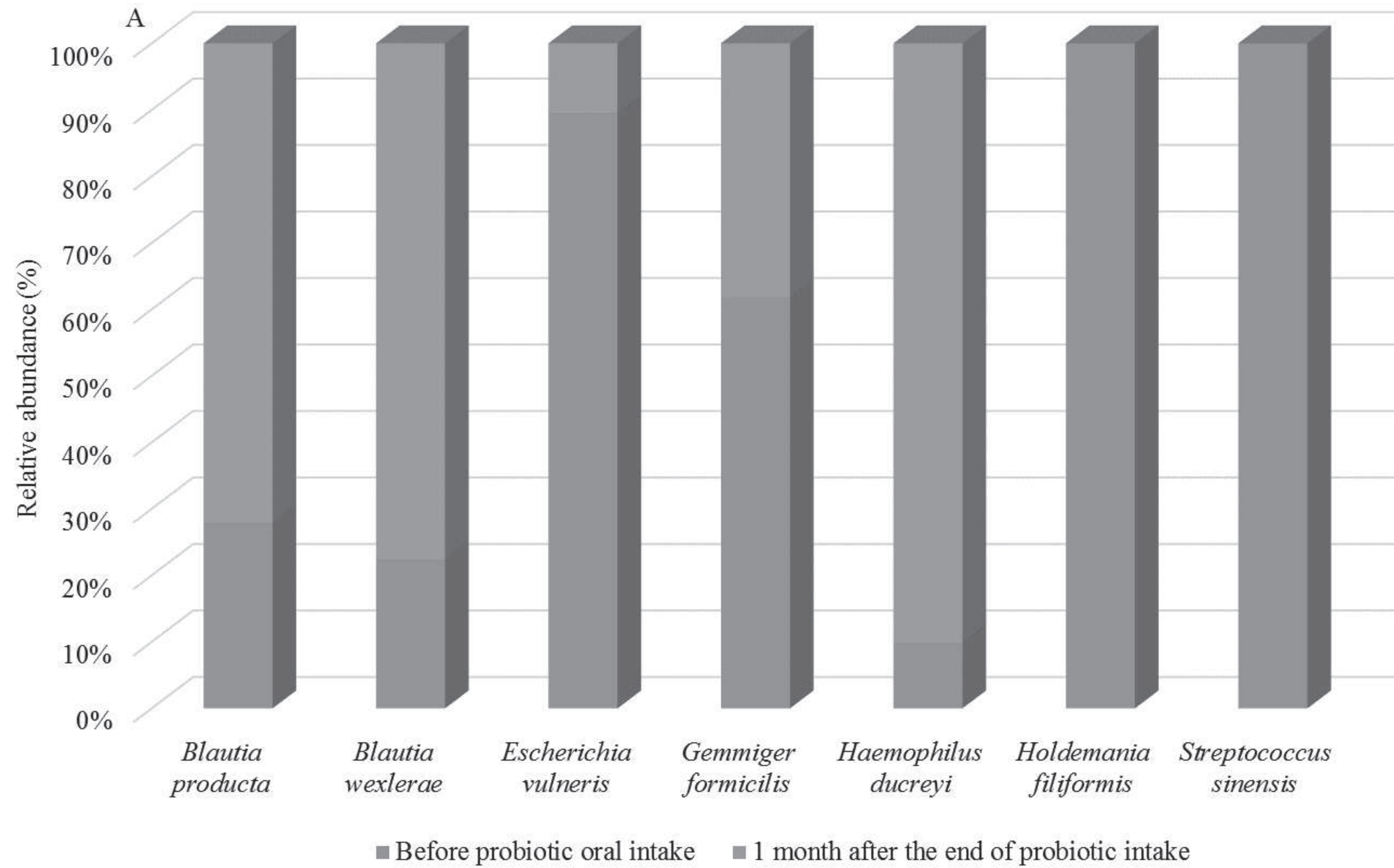
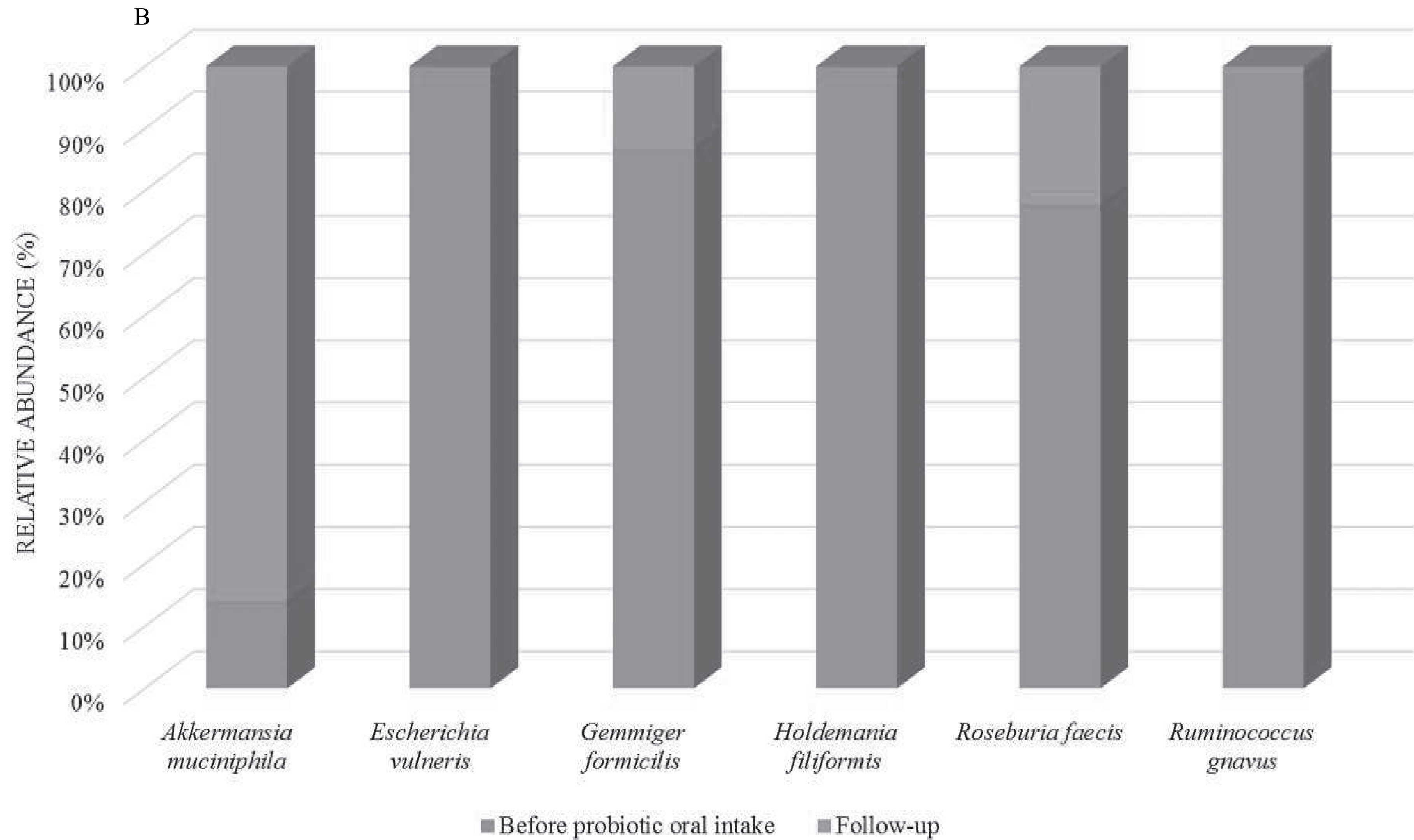


Figure 4



Manuscript 5

Microbiota network and mathematic microbe mutualism in colostrum and mature milk collected in two different geographic areas: Italy versus Burundi

Published in The ISME Journal

Introduction

The aim of this study was to investigate the bacterial composition of colostrum and mature milk in mothers belonging to genetically different populations living in two geographically and culturally different countries, such as Italy and Burundi. In particular, we integrated Auto CM to metagenomics analysis and investigated the potential bacterial interactions in biological samples. Traditional analysis methods, such as Hierarchical clustering and PCA, were performed to evaluate the bacterial composition in aforementioned groups. Since the results obtained by the traditional benchmarking methods were not significant, they were not included in the manuscript but, for further information, they were reported in the present PhD work.

Main Results

The human milk microbiota is a dynamic and complex ecosystem whose bacterial interactions contain diverse microbial hubs and central nodes which change during the transition from colostrum to mature milk. Moreover, a greater abundance of anaerobic intestinal bacteria in mature milk compared with colostrum samples has been observed.

Breakthrough

The results of hierarchical clustering analysis were obtained calculating a distances matrix based on the Jaccard coefficient. The emerging information is rather poor and the main difficult is organizing the clustering in terms of direct associations with a global view. Similarly, also the results obtained with Principal Component Analysis gave a too complex view of the microbial system under investigation.

Indeed, not only the visualization of all microorganisms' labels was not available but, as previously encountered in the hierarchical clustering, a systemic view of the bacterial network was not considered. An example of results obtained by Hierarchical clustering and PCA are reported in **Appendix** (Figure 1 and 2, respectively).

Differently, using the Auto CM, we highlighted that human breast milk has a dynamic bacterial composition, which changes at quantitative and qualitative level (bacterial abundance and microbiota network) during its transition from colostrum to mature milk. In particular, combining the classic abundance analysis with the study of bacterial networks, we can define and deeply characterize the bacterial populations associated to any biological samples.

OPEN

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ORIGINAL ARTICLE

Microbiota network and mathematic microbe mutualism in colostrum and mature milk collected in two different geographic areas: Italy versus Burundi

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Human milk is essential for the initial development of newborns, as it provides all nutrients and vitamins, such as vitamin D, and represents a great source of commensal bacteria. Here we explore the microbiota network of colostrum and mature milk of Italian and Burundian mothers using the auto contractive map (AutoCM), a new methodology based on artificial neural network (ANN) architecture. We were able to demonstrate the microbiota of human milk to be a dynamic, and complex, ecosystem with different bacterial networks among different populations containing diverse microbial hubs and central nodes, which change during the transition from colostrum to mature milk. Furthermore, a greater abundance of anaerobic intestinal bacteria in mature milk compared with colostrum samples has been observed. The association of complex mathematic systems such as ANN and AutoCM adopted to metagenomics analysis represents an innovative approach to investigate in detail specific bacterial interactions in biological samples.

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Introduction

Breastfeeding is the first form of infant feeding, as it allows newborns to assimilate all the nutrients needed in the first 6 months of life (González *et al.*, 2013). Essential for initial development (Fernández *et al.*, 2013), human milk is able to influence the development of the immune system and helps establish gut microbiota by its components, such as oligosaccharides, acting naturally as prebiotics (Fernández *et al.*, 2013).

The composition of human milk differs according to the timing of lactation. Colostrum is generated immediately after delivery until the fifth/sixth day of life and is very rich in nutrients and bioactive factors, containing proteins, mineral salts, oligosaccharides, antibodies, cytokines, lysozyme and complement factors (Ballard and Morrow, 2013). From 5 days to 2 weeks postpartum, the transitional milk occurs (Ballard and Morrow, 2013). Its function is to support the nutritional and developmental needs of growing infants, and for this reason, it is rich in lactose, calcium, lipids and glucids (Ballard and Morrow, 2013). One month after childbirth, the human milk achieves a standard composition, known as 'mature milk'. This is characterized by a lower percentage of proteins and minerals and a greater richness in lipids and carbohydrates compared with colostrum (Ballard and Morrow, 2013).

In the past decade, there has been an increased interest in the study of microbiota in human milk. More than 200 different species belonging to 50 different genera have been described in human milk samples (Hunt *et al.*, 2011). Today it is recognized as a potential source of probiotic bacteria, such as streptococci, lactobacilli and bifidobacteria (Martín *et al.*, 2003), which have been observed to have a pivotal role in the first stage of initial neonatal gut colonization (Fernández *et al.*, 2013), allowing babies to ingest between 1×10^5 and 1×10^7 bacteria daily (Heikkilä and Saris, 2003).

The mature milk microbiota appears around 1 month after delivery, being influenced by hormonal signaling, lifestyle and diet. This results in significant

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changes to the gut microbiota of pregnant woman (Chapman and Nommsen-Rivers, 2012) in different geographic areas.

The human milk microbiota comes from several body sites, probably including the maternal gut microbiota (Fernández *et al.*, 2013), through an entero-mammary pathway, as well as a specific degree of retrograde flow back into the mammary ducts that can occur during nursing (Hunt *et al.*, 2011). It has been hypothesized that intestinal bacteria could translocate to the mother's blood stream, reaching the mammary ducts. However, to date it is not clear how and when intestinal microorganisms colonize the mammary epithelium. Interesting, mature milk bacteria have been observed to reduce respiratory and diarrheal infections in early infancy, having also a protective role toward breastfeeding women (Fernández *et al.*, 2013; Bergmann *et al.*, 2014).

The aim of this study was to evaluate the microbiota network, and differences, of colostrum and mature milk in mothers living in two completely different sites and environments, such as Italy and Burundi. In addition, we performed a data-mining evaluation with advanced complex mathematic systems to assess the pattern of commensal clustering using the auto-contractive map (AutoCM).

Subjects and methods

Study population

This study involved the mother and newborn pairs recruited from hospital postnatal wards in the Republic of Burundi (Hospital of Ngozi) and in Verona, Italy (Policlinico GB Rossi).

Participants were selected if they met the following inclusion criteria: (a) healthy infants, (b) willingness to comply with study protocol, and (c) postpartum colostrum collection within 3 days of birth. Exclusion criteria included: (a) significant maternal or infant illness or major birth defects, (b) mothers taking immunosuppressive agents, and (c) mothers taking antibiotic during lactation.

Mothers in Verona represent a western population living in an environment typical of the European countries. Their diet was rich in calories, including high rate of animal proteins, sugar and fat and low rate of fibers, vegetables and fruits. In contrast, the mothers from Burundi live in small villages and came to the hospital just for delivering. The Burundian diet of participant mothers living in these small rural villages consisted mainly of cereals, legumes and vegetables, being rich in fibers and poor of animal proteins, animal fats and sugar.

The sample collection and investigation were conducted following ethical approval by separate committees in the two participating hospitals in accordance with Italian standards (Ethical Committee of the Azienda Ospedaliera di Verona, Italy, Approval No. 1288).

Informed consent was obtained from all subjects.

Collection and processing of milk samples

One sample of colostrum and one sample of mature milk was collected from each mother.

Colostrum was collected within 3 days postpartum, and mature milk at 1 month of life. All mothers were given two sterile plastic tubes in which they collected colostrum and mature milk. Samples were collected after the mother's hands and areola area were cleaned using a preservative-free soap to allow a deep bacterial decontamination. Samples were initially stored in a fridge and frozen to $-20\,°C$ within 2 h of expressing. Samples from Burundi were transported to Verona under controlled conditions. Within the laboratories in Verona, the samples were thawed and transferred into plastic microcentrifuge tubes (Eppendorfs, Milan, Italy) and centrifuged at 1500 g for 15 min at 4 °C to separate the fat and the aqueous phase.

Bacterial DNA extraction and 16S gene sequencing

Total DNA was extracted from colostrum and mature milk samples using a Milk DNA Extraction Kit (Norgen, Thorold, Ontario, Canada) following the manufacturer's instructions. The protocol included the specific binding of DNA to the QIAmp silica-gel membrane while contaminants pass through (Salonen *et al.*, 2010). DNA amplification and gene sequencing were performed as previously described (Drago *et al.*, 2016).

The auto-contractive map

System biology inherent to human–microbe mutualism is related to the collection of large amounts of data per single subject, and complex mathematical networks can help us in establishing the hierarchy of variables within a specific set. We have adopted the AutoCM to illustrate this.

AutoCM system is a fourth-generation unsupervised artificial neural network (ANN), which has already been demonstrated to outperform several other unsupervised algorithms in a heterogeneous class of tasks (Buscema and Sacco, 2016). AutoCM is able to highlight the natural links among variables with a graph based on minimum spanning tree theory, where distances among variables reflect the weights of the ANN after successful training phase (Buscema and Grossi, 2008a; Buscema *et al.*, 2008b; Buscema and Sacco, 2010). The AutoCM system finds, by a specific learning algorithm, a square matrix of 'similarities' (weights mathematically speaking) among the variables (in this case, microbes' abundances) of the data set. Once the AutoCM weights' matrix is obtained, it is then filtered by a Minimum Spanning Tree (MST) algorithm (Kruskal, 1956; Fredman and Willard, 1990). MST shows among the huge number of possible ways to connect the variables in a tree, the shortest possible combination. In the MST, in fact, every link able to generate a cycle into the graph is eliminated, irrespective of its

strength of association, and this results in a simplified graph. A graphical description of MST concept is provided in Supplementary Appendix S1. The assumption is that as all biological systems tend naturally to the minimal energetic states this graph express the fundamental biological information of the system. The ultimate goal of this data mining model is to discover hidden trends and associations among variables, as this algorithm is able to create a semantic connectivity map in which non-linear associations are preserved and explicit connection schemes are described. This approach shows the map of relevant connections between and among variables and the principal hubs of the system. Hubs can be defined as variables with the maximum amount of connections in the map.

The learning algorithm of CM may be summarized in four orderly steps: (a) signal transfer from the input into the hidden layer; (b) adaptation of the connections value between the input layer and the hidden layer; (c) signal transfer from the hidden layer into the output layer; and (d) adaptation of the connections value between the hidden layer and the output layer.

The AutoCM neural networks does not have initial weights posed at random. They start always by the same value. Therefore, the resulting graph is perfectly reproducible along many possible runs.

A detailed description of the theory, mathematics and functioning of this analytical technique is provided in Supplementary Appendix 2.

In simple words, AutoCM 'spatializes' the correlation among different variables ('closeness') and converts it into a compelling graph that identifies only the relevant associations and organizes them into a coherent picture, building a complex global picture of the whole pattern of variation. We choose arbitrarily to consider as relevant hubs those microorganisms that showed at least five connections with other microorganisms in the network. Moreover, we defined a 'central node' the inner node that is the last remaining after bottom-up recursively pruning away the 'leaves' nodes (that is, the isolated ends of the graph).

Results

Study population

The study was proposed to 40 mothers in Italy and in 40 in Burundi. A total of 50 mothers were included in this study (20 mothers from Italy and 30 from Burundi), providing consent to participate and presenting characteristics according to the inclusion criteria. From all Italian subjects, we obtained colostrum and mature milk samples, while only 12 of the Burundian mothers (owing to the local habits) provided the mature milk samples. Demographic details of the two populations are shown in Table 1, where also characteristics of the 12 Burundian pairs who provided both colostrum and mature milk are shown.

Bacterial abundance in colostrum and mature milk samples

Colostrum and mature milk of both populations showed a high bacterial abundance, as >200 bacterial genera have been detected in all samples (Figure 1). The main bacterial genera found in each group are summarized in Table 2 (each bacterial genus represents at least the 2% of total bacteria contained in samples).

The auto-contractive map

We used the AutoCM to represent the main connections between all bacterial genera found in colostrum and mature milk samples of both populations. This includes all the variables considered linked in a way that the energy structure of the system is minimized or, if weights are correlation measures, in a way that these are maximized for all the connections in the graph.

The MST algorithm does not map all the correlations present in the data but only the strongest

Table 1 Demographic characteristics of mother–newborn pairs from the two different sites

		Italy 20 pairs	*Burundi 30 pairs*	P-*values B vs I*	*Burundi 12 pairs (C+M)*
Maternal age (years)	Mean (± s.d.)	35.7 (±5.54)	23.4 (±5.40)	<0.001	24.5 (±5.80)
Gestational age (weeks)	Mean (± s.d.)	39.2 (±1.35)	37.2 (±1.20)	<0.01	37.5 (±1.38)
Gender (male)	*N* (%)	12/20 (60)	18/30 (60)	NS	7/12 (58)
Mode of delivery (c-section)	*N* (%)	4/20 (20)	5/30 (16)	NS	3/12 (10)
Previous deliveries	Mean (± s.d.)	0.84 (±0.80)	2.01 (±1.10)	<0.05	2.14 (±1.38)
Active smoking	*N* (%)	2/20 (10)	3/30 (10)	NS	1/30 (3)
Passive smoking	*N* (%)	8/20 (40)	3/30 (10)	<0.05	1/30 (3)
Antenatal antibiotic	*N* (%)	0/20 (0)	5/30 (16)	<0.02	3/30 (10)
Antenatal infection	*N* (%)	6/20 (30)	13/30 (43)	NS	6/30 (20)
Perinatal antibiotics in mothers	*N* (%)	4/20 (20)	5/30 (16)	NS	3/30 (10)
Perinatal antibiotics in offsprings	*N* (%)	2/20 (10)	0/30 (0)	NS	0/30 (0)

Abbreviations: B, Burundi; C, colostrums; c-section, cesarean section; I, Italy; M, mature milk; NS, not significant.
No significant difference was obtained comparing the whole group of 30 Burundi pairs and the 12 pairs whose mothers provided both colostrum and mature milk.

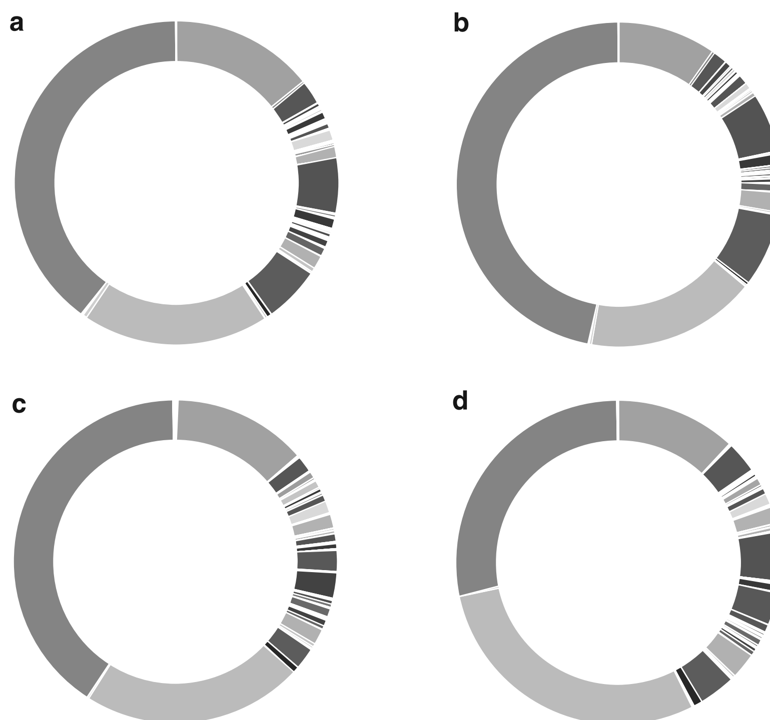

Figure 1 Bacterial distribution in each type of milk sample: (**a**) Italian colostrum; (**b**) Italian mature milk; (**c**) Burundian colostrum; (**d**) Burundian mature milk.

correlations between the hubs in the system that are connected to a node and only a single path is available between two hubs (no loops). Several bacterial hubs were observed in all samples, and interestingly, the aforementioned hubs were different between all groups analyzed.

In the Italian colostrum, the main bacterial hubs were represented by *Abiotrophia* spp, *Actinomycetospora* spp, *Aerococcus* spp, *Alloiococcus* spp, *Amaricoccus* spp, *Bergeyella* spp, *Citrobacter* spp, *Desulfovibrio* spp, *Dolosigranulum* spp, *Faecalibacterium* spp, *Parasutterella* spp, *Rhodanobacter* spp and *Rubellimicrobium* spp. *Abiotrophia* spp, in particular, represented the biggest hub of the entire network as it had 59 connections to other microorganisms (Figure 2).

Figure 1 is obtained from a data set composed by 20 rows (subjects) and 269 columns (microbes abundances). Therefore, the graph expresses the overall schema of microbes' association in the observed sample. The same concept applies to other figures.

Moreover, *Aciditerrimonass* spp represented the central node of the bacterial network.

The Italian mature milk instead shared with colostrum samples only *Abiotrophia* spp and *Aerococcus* spp presenting other bacterial hubs, such as *Acetanaerobacterium* spp, *Aciditerrimonas* spp, *Acidocella* spp, *Aminobacter* spp, *Bacillus* spp, *Caryophanon* spp, *Delftia* spp, *Microvirga* spp, *Parabacteroides* spp and *Phascolarctobacterium* spp (Figure 3). *Alistipes* spp was the central node of network.

Furthermore, bacterial hubs observed in Burundian colostrum were *Aeribacillus* spp, *Agaricola* spp, *Alterythrobacter* spp, *Amaricoccus* spp, *Aquabacterium* spp, *Aquimonas* spp, *Brachybacterium* spp, *Dolosigranulum* spp, *Micrococcus* spp, *Peptostreptococcus* spp, *Propionibacterium* spp and *Serratia* spp (Figure 4), while in Burundian mature milk *Achromobacter* spp, *Aeromicrobium* spp, *Aggregatibacter* spp, *Albidovolum* spp, *Aquipuribacter* spp, *Aurantimonas* spp, *Bergeyella* spp, *Buttiauxella* spp, *Dolosigranulum* spp, *Parasutterella* spp, *Tepidiphilus* spp and *Weissella* spp represented

Table 2 Main bacterial genera detected in colostrum and mature milk of Italian and Burundian populations

Colostrum (Italy)	*Mature milk (Italy)*	*Colostrum (Burundi)*	*Mature milk (Burundi)*
Achromobacter	*Achromobacter*	*Achromobacter*	*Achromobacter*
Acinetobacter	*Acinetobacter*	*Acinetobacter*	*Acinetobacter*
Bacteroides	*Agrobacterium*	*Alcaligenes*	*Delftia*
Corynebacterium	*Corynebacterium*	*Arthrobacter*	*Enterococcus*
Delftia	*Delftia*	*Corynebacterium*	*Gemella*
Flavobacterium	*Flavobacterium*	*Delftia*	*Halomonas*
Gemella	*Gemella*	*Enterococcus*	*Klebsiella*
Halomonas	*Halomonas*	*Gemella*	*Pseudomonas*
Prevotella	*Propionibacterium*	*Klebsiella*	*Rhizobium*
Propionibacterium	*Pseudomonas*	*Leuconostoc*	*Rothia*
Pseudomonas	*Rhizobium*	*Pantoea*	*Serratia*
Rhizobium	*Rothia*	*Pseudomonas*	*Staphylococcus*
Rhodanobacter	*Staphylococcus*	*Rhizobium*	*Streptococcus*
Rothia	*Streptococcus*	*Rothia*	
Serratia		*Serratia*	
Staphylococcus		*Staphylococcus*	
Stenotrophomonas		*Streptococcus*	
Streptococcus			

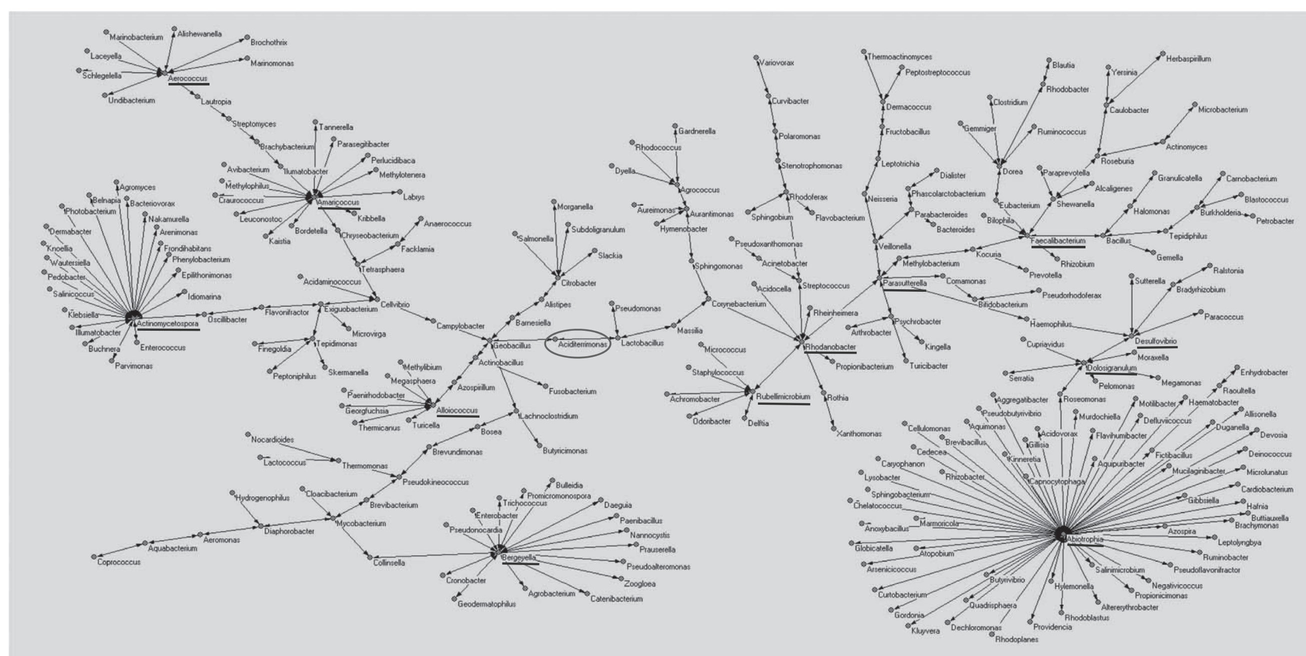

Figure 2 Microbiota network of Italian colostrum. The main hubs of the bacterial network are underlined with a blue line; red circle shows the central node of the network.

the main bacterial hubs (Figure 5). *Sphingomonas* spp and *Rhizobium* spp represented the central nodes of Burundian colostrum and mature milk, respectively. Interestingly, in all groups bacterial hubs did not coincide with the main bacterial genera found in samples, except for *Achromobacter* and *Rhizobium* in Burundian mature milk. A detailed description of the theory, mathematics and functioning of this analytical technique has been provided in Supplementary Appendix.

Furthermore, in colostrum and mature milk of both populations we found a high prevalence of anaerobe bacteria (Figure 6a) and lactic acid bacteria (Figure 6b).

Discussion

This is the first study in which the microbiota of colostrum and mature milk of Italian and African populations has been compared.

The development of cultivation-independent techniques for the study of bacterial population of different biological samples allows a deeper analysis of bacterial diversity existing in aforementioned samples. Dietary habits are considered one of the main factors influencing the human microbiota composition, as the intake of meat, vegetables, proteins and fibers leads to significant changes in human-associated bacterial diversity (De Filippo *et al*., 2010). Western and African lifestyle

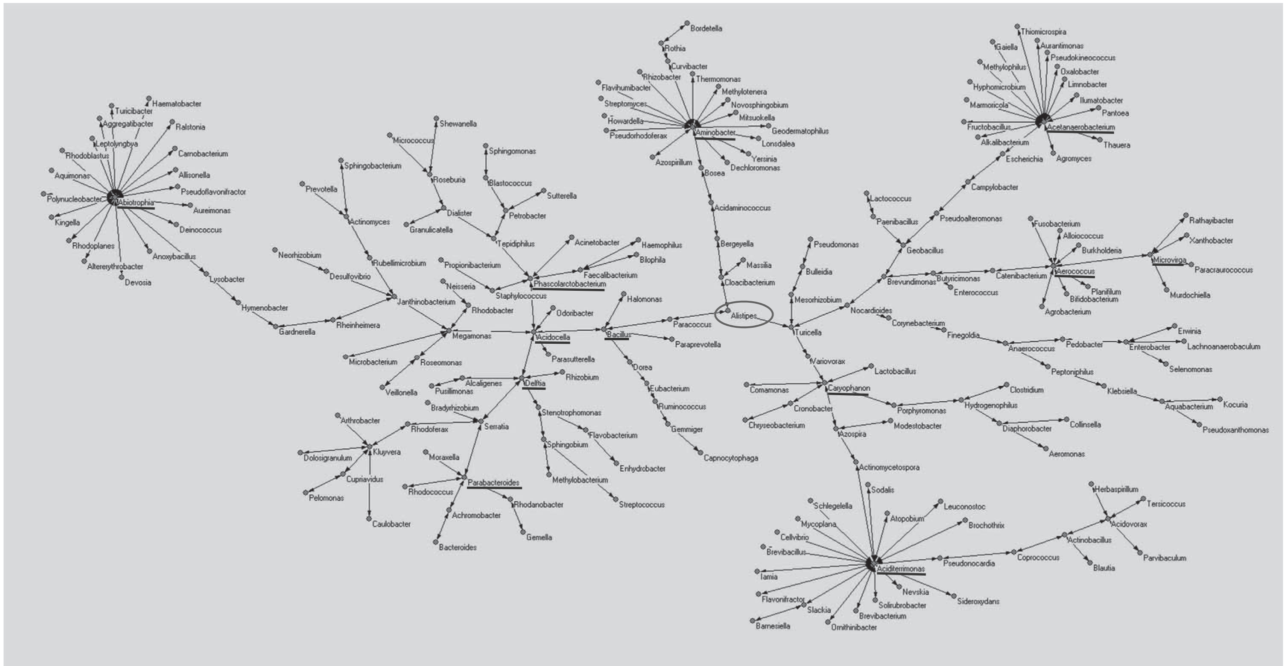

Figure 3 Microbiota network of Italian mature milk. The main hubs of the bacterial network are underlined with a blue line; red circle shows the central node of the network.

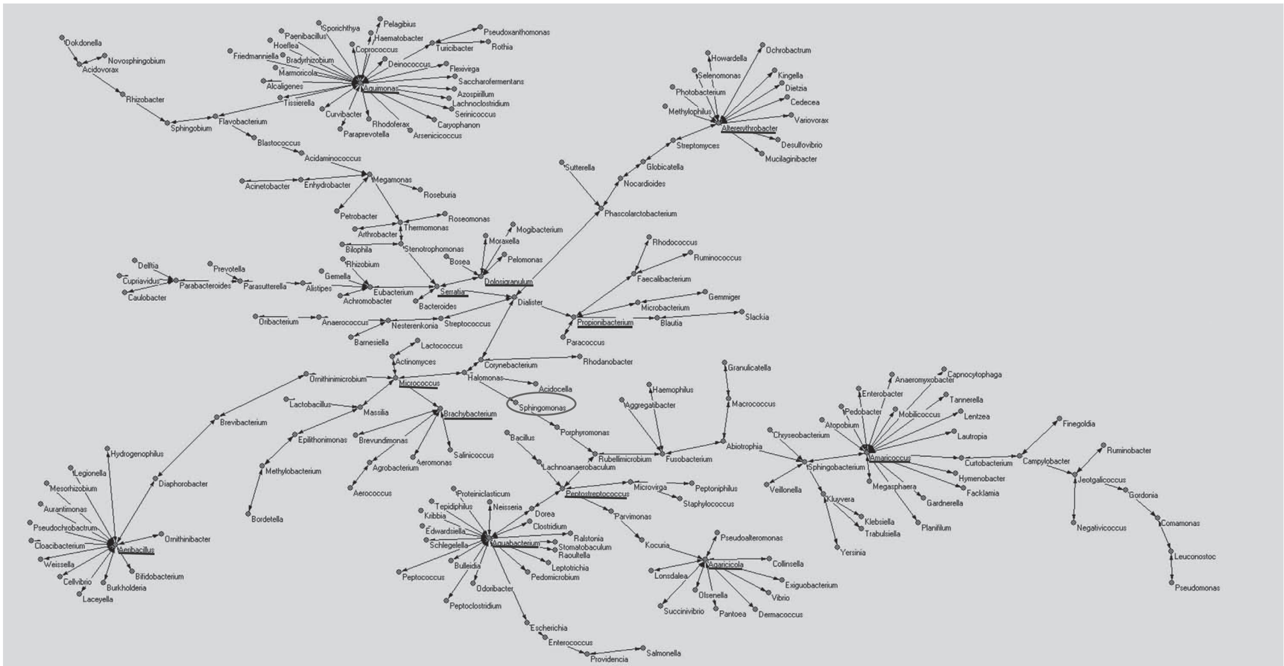

Figure 4 Microbiota network of Burundian colostrum. The main hubs of the bacterial network are underlined with a blue line; red circle shows the central node of the network.

and diet are very different, and for this reason, bacteria specialized in human-associated niches underwent deep modifications during the social and demographic changes (De Filippo *et al*., 2010).

Even though we have not investigated the diet habits of our two populations by a food-frequency questionnaire, we assumed that mothers in the two groups represented two completely different environments. Indeed, the mothers in Verona represent a western population living in an environment typical of the developed world, following a diet rich in animal proteins, sugar and fat and low in fibers. Mothers from Burundi, instead, followed a very different diet if compared with the western one, as it

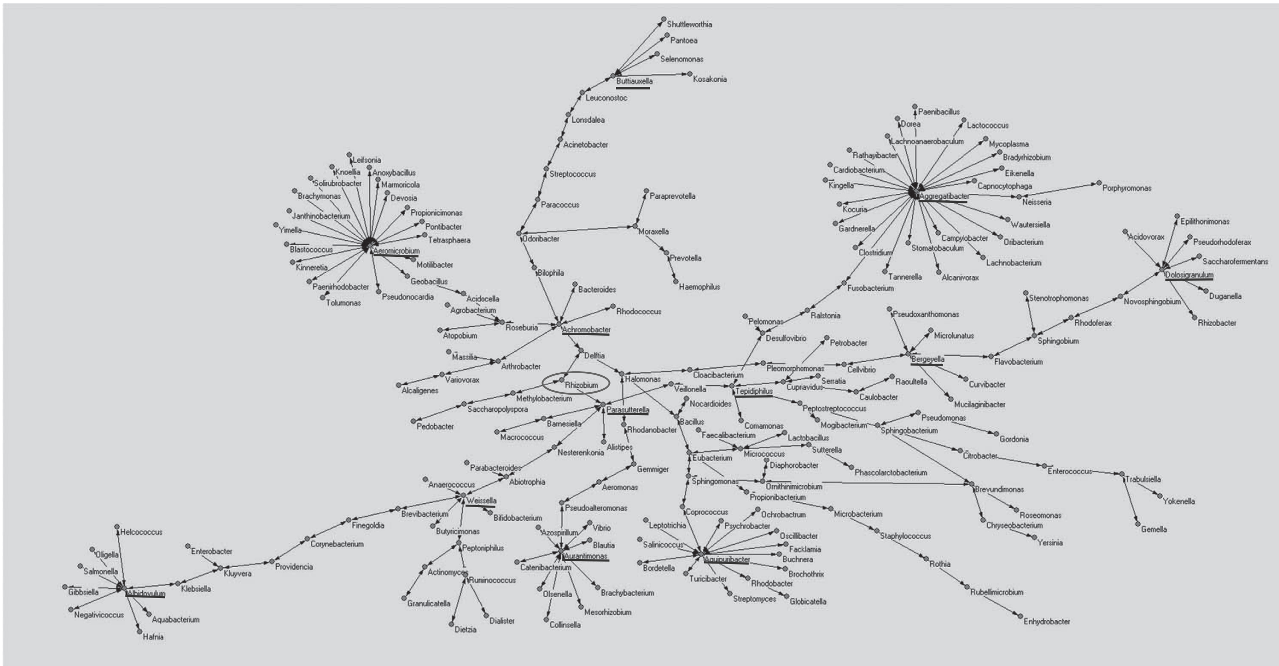

Figure 5 Microbiota network of Burundian mature milk. The main hubs of the bacterial network are underlined with a blue line; red circle shows the central node of the network.

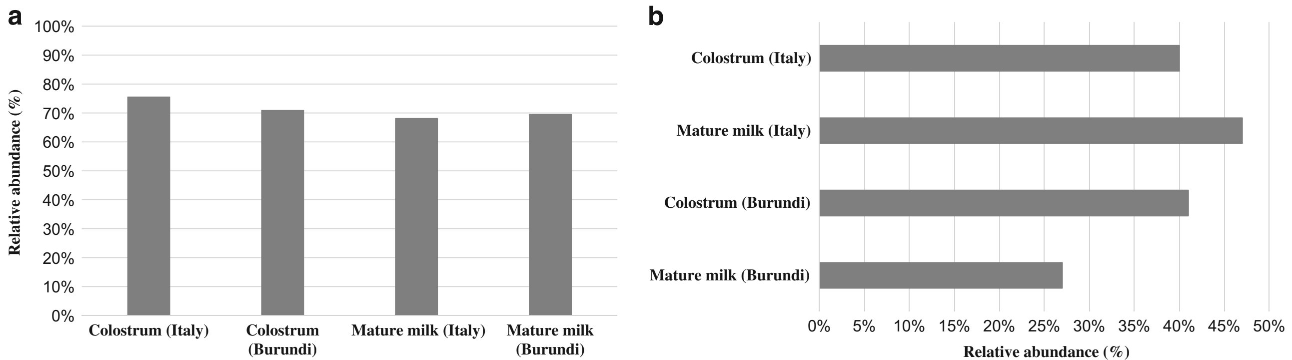

Figure 6 (**a**) Relative abundance of anaerobe bacteria in both Italian and Burundian colostrum and mature milk; (**b**) Relative abundance of lactic acid bacteria in both Italian and Burundian colostrum and mature milk.

was characterized by high proportions of cereals, legumes and vegetables, being rich in fibers and poor in animal proteins and sugar.

All samples of colostrum and mature milk from Italy and Burundi showed different bacterial distributions in the microbiota network. Synergy is a positive interaction between bacterial species or strains that leads to several benefits and advantages to microorganisms. Consequently, bacteria are not randomly distributed throughout the body but their specific localization and, above all, their reciprocal interactions, are essential for their survival in the human body (Stacy *et al.*, 2016).

Abiotrophia spp represent a main hub found only in colostrum and mature milk from Italian mothers. This bacterial genus is a nutritionally variant of streptococci originally isolated from patients with endocarditis and otitis media (Roggenkamp *et al.*, 1998). It is a common microorganism belonging to the microbiota of oral cavity, genitourinary tract and gastrointestinal tract, which has never been associated with human bacteremia; consequently, its presence in human milk may be the direct consequence of a retrograde flow back into the mammary ducts that can occur during nursing (Hunt *et al.*, 2011) or the entero-mammary pathway (Fernández *et al.*, 2013).

Several findings have highlighted the ability of maternal intestinal bacteria to reach the mammary glands by mean of dendritic cells and CD18$^+$ cells, which would bind to non-pathogenic microorganisms from the gut lumen, carrying them to lactating

mammary gland (Rodriguez, 2014). The high percentage of anaerobe intestinal microorganisms found in colostrum and mature milk of both Italian and Burundian populations may support the hypothesis of entero-mammary pathway.

Colostrum from Italian mothers was also rich in lactic acid bacteria, such as *Alloiococcus* spp, which represent one of the main hubs found in this biological sample. This bacterial genus is a common member of the vaginal microbiota and contributes to the balance between beneficial and pathogenic bacteria in the vaginal ecosystem, providing protection against harmful microorganisms (Martín *et al.*, 2003; Ling *et al.*, 2010). Consequently, it may act as a protective microorganism in newborns, as breastfeeding protects babies against several diseases, not only owing to immunological contents of human milk, such as immunoglobulins and immunocompetent cells, but also by means of probiotic bacteria that reach a high concentration in this biological sample (Gilliland, 1990). Lactic acid bacteria also has numerous beneficial properties in the human organism as they are able to control intestinal infections, improve nutritional value of food and stimulate the immune system (Gilliland, 1990).

In contrast, in the mature milk from Italian mothers *Parabacteroides* seem to have a pivotal role in the bacterial network. *Parabacteroides* spp are intestinal microorganisms and their presence in the human milk could be explained by the aforementioned entero-mammary pathway. Interestingly, *Parabacteroides* is able to produce bacteriocins that inhibit the RNA synthesis, without effect on protein, DNA or ATP synthesis (Nakano *et al.*, 2006). These particular peptides exert a broad-spectrum of antimicrobial activity, and their activity is not linked to the development of resistance in target bacteria (Nakano *et al.*, 2006). As a consequence, the ability of *Parabacteroides* to produce bacteriocins allows not only the establishment of this microorganism in the mammary gland ecosystem but also the control of excessive growth of potential pathogenic microorganisms.

Furthermore, an *in vivo* study showed that the administration of *Parabacteroides distasonis* antigens was able to reduce the impact of intestinal inflammation in animal models of colitis, highlighting the potential protective role of *Parabacteroides* spp in the host's organism (Kverka *et al.*, 2011). Also *Phascolarctobacterium* seem to have a pivotal role in Italian mature milk. This particular microbial genus is a common member of the *Firmicutes* phylum and it produces high amount of the short-chain fatty acids acetate and propionate, which stimulate colonic blood flow and electrolyte uptake and act as energy source for muscles (Topping and Clifton, 2001).

Conversely, in Burundian colostrum the majority of bacterial hubs are represented by potential pathogens such as *Serratia* spp and *Peptostreptococcus* spp or poor-characterized microorganisms, of which the biological role in the human organism is still the object of study. The bacterial genus *Aquabacterium*, which seems to have a central role in Burundian colostrum, has been described as a colonizer of the very premature infant gut dominant microbiota (Aujoulat *et al.*, 2014). Not surprisingly, this specific genus does not have a further role in the mature milk from Burundian mothers. In our study, we observed that *Aquabacterium* was connected with 18 different bacterial genera, belonging above all to the intestinal microbiota, but to date the real biological meaning of these interactions are unknown. It is interesting to underlie that the central hub of the microbiota network in mature milk from mothers in Burundi is *Rhizobium*. As this is a soil bacterium that is a symbiont of the legumes, and not a documented gut microbe, it is tempting to speculate that it possibly enters milk from the large amounts of legumes that we know these mothers consume through the entero-mammary pathway. Unfortunately, mothers enrolled in our study were not subjected to a food-frequency questionnaire, and consequently, we have not detailed information about their diet to advance more specific hypothesis about the relationship between the detection of *Rhizobium* in milk samples and food intake.

Interestingly, Salter *et al.* (2014) demonstrated that numerous laboratory reagents and DNA extraction kits are contaminated with bacterial DNA, which can negatively influence the results of metagenomics study, above all when analyzing samples containing low microbial mass (Salter *et al.*, 2014). As *Rhizobium* belongs to the potential DNA kit contaminants found by Salter *et al.* (2014), it is reasonable thinking that its presence in milk samples may be the result of DNA extraction kit contamination. However, in the present study a negative control (no DNA sample) was added during bacterial DNA amplification to verify the absence of any contamination. No amplification products have been observed, indeed the agarose gel showed no amplification bands and the DNA quantification with Qubit provided no detectable DNA in negative control, leading us to hypothesize that the presence of *Rhizobium* in our samples did not derive from sample contamination during processing. Moreover, the detection of *Rhizobium* as central node only in African mature milk and not in Italian one strengthen our hypothesis that its detection is closely related to plant- and legume-rich diet generally followed by Burundian population.

Moreover, *Dolosigranulum* spp, which is the only bacterial hub shared with Burundian mature milk, showed having a protective role in the host's organism. *Dolosigranulum* and *Streptococcus pneumoniae* seem to be involved in a competitive interaction that would inhibit the pathogenesis of otitis media by the pathogenic activity of *S. pneumoniae* (Fusco *et al.*, 2015).

Finally, in the mature milk collected from Burundian mothers *Dolosigranulum* and *Weissella* may have a protective role against infections and pathogenic bacteria

in both mothers and newborns. This microorganism is a lactic acid bacterium often isolated from human skin, feces, saliva and milk and from African traditional fermented foods (Fusco *et al*., 2015). Some *Weissella* strains may have a probiotic activity, as they are able to inhibit *in vitro* biofilm formation and the proliferation of *Streptococcus mutans*, which is often involved in dental caries (Fusco *et al*., 2015). The ability of *Weissella* to produce bacteriocins and exert an *in vitro* anti-inflammatory activity in human mouth epithelial cells elicited by *Fusobacterium nucleatum* strengthen the hypothesis that this microorganism may have a high probiotic potential (Kang *et al*., 2006; Papagianni and Papamichael, 2012; Papagianni and Sergelidis, 2013).

In conclusion, in our study we observed several differences in the microbiota network of colostrum and mature milk from Italian and Burundian mothers. Bacterial relations changed within the same population, underlying that colostrum and mature milk are different not only for protein and fat content but also for the microbiota composition. We believe some bacterial genera are essential in the first phase of lactation, and for this reason, they have a pivotal role in colostrum, while other microorganisms are fundamental in the long-term nutrition of newborns, having consequently a major role in mature milk.

Our study highlighted the impact that lifestyle and dietary habits may have on the microbiota composition of human milk, being so different between Italy and Burundi, and diet foremost might explain the major differences in the microbiome composition and network. Nevertheless, at the same time we must consider that, besides lifestyle and dietary habits, there are a number of other differences between these two populations that may influence the findings. In particular, in Burundi mothers are younger, their babies are born earlier, they were more likely to have had previous deliveries, they are less likely to be exposed to second-hand smoke and are more likely to have had antenatal antibiotics. Consequently, it is necessary to consider that different factors can contribute to modulate the human milk microbiota. Out of them, probably specific foods or food supplements may represent a more direct and sustainable strategy to protect newborns from the onset of several infections and diseases, promoting the growth of probiotic and beneficial bacteria with a protective role for the host.

To date, the real biological meaning of many bacterial hubs found in the microbiota network are not clear and they are still the object of studies, even if the mathematical model we applied demonstrated that they are probably fundamental for maintaining the microbiota homeostasis and that their breakdown could be responsible of a probable ecosystem unbalance.

The techniques used to illustrate the association between the bacteria is novel and therefore their properties and implications are not currently entirely understood, and further research is called for to explore them. Any way, the disappointing results obtained with two alternative data mining statistical methods such as hierarchical clustering and principal component analysis strengthen the idea that AutoCm, thanks to its new sophisticated mathematics, could become, in the future, a reference approach to better understand the complexity of human–microbe mutualism.

Similarly, further studies are needed to better characterize all interactions between the bacterial hubs and the branches of microbiota network observed in the present study, in order to define the specific biological meaning of bacterial distribution in human milk samples.

Conflict of Interest

The authors declare no conflict of interest.

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Chapter 5

DISCUSSION

During the last decades, many studies were performed to investigate and elucidate human microbiota composition and its interactions with the host. Today, it reasonably claiming that we are living in the "Human microbiome golden age" since, thanks to scientific initiatives, such as Metagenomics of Human Intestinal Tract (MetaHIT) project in Europe and Human Microbiome Project (HMP) in U.S.A, there is a huge amount of information that increases the interest of researchers to understand how microbial communities respond to perturbations. However, to date it is extremely difficult translating such vast potential in clinical application. The present PhD study arises from the will in contributing, in future, to translate microbiome researches into real applications for clinical microbiology.

In all studies performed during PhD (related papers are attached to the present thesis), a metagenomics approach was applied to characterize the human microbiota associated to different kind of samples and different experimental conditions, using the Ion Torrent ™ PGM. Undoubtedly, 16S rDNA gene sequencing allows us to highlight a great diversity and distribution of microbial communities in contrast to traditional cultured–dependent methods, that were recently dismissed in our laboratory. In particular, to compare our study groups, we used mainly two setups: parallel and cross-over comparison. In the first case, following a specific experimental time course, for each individual belonging to a unique population, two or more samples were obtained at different time-points and subsequently compared with each other. Consequently, potential intra-population effects should have been minimized. In cross-over study design, instead, samples collected from two or more groups of participants belonging to different populations were compared. Therefore, inter-population differences could have a deep impact on experimental outcome. Nevertheless, in all our studies we found many difficulties to analyze data obtained both from cross-over and parallel comparisons. In particular, this was due to the high individual variability that characterizes human microbiota. In our first work (Manuscript 1), we tried to identify differences in microbial community composition by comparing only bacterial relative abundance. Analyzing our data superficially at family level, we understood how extremely heterogeneous metagenomics data are.

Although several significant differences were observed, a clear disadvantage in this study, as well as in subsequent ones, was the small number of participants. However, in metagenomics studies the high sample heterogeneity remains an unresolved issue, even if a larger number of samples was available. In particular, data generated from different studies involved in microbiota research are not still optimally comparable. This is due mainly to the lack of reference materials, such as standard samples and procedures shared in the scientific community. Moreover, along every step of a metagenomics experimental protocol, many biases can be introduced, from sample collection to data analysis and interpretation. For example, different microbiota studies are performed using different experimental approaches and analytical pipelines, leading to confounding results [35]. Furthermore, it is often very difficult collecting detailed metadata providing information on patient's health, including dietary intake or personal habits. Consequently, attempts to compare datasets generated from different cohorts may become extremely confusing. In addition, in literature numerous researches highlighted the existence of a huge amount of factors that can deeply influence the microbiota composition, including exposure to sunlight, jet lag, seasonality and circadian rhythms [36]–[39]. However, to date many of these elements are very difficult to keep in consideration and survey especially during the collection of clinical samples and subjects' data. For this reason, in our second study (Manuscript 2), we tried to reduce some of potential confounding factors, introducing strict inclusion and exclusion criteria before sampling, regarding sexual activities, clothing and personal care. Although at superficial level (bacterial family) we found many significant differences in skin microbiota composition of subjects enrolled in this study, our data showed that a more deepen survey of microbial taxa composition (genus and species level) may better underline differences between individuals belonging to the study population. This latter strategy is particularly useful when we want emphasizing specific correlations or interplays between the microbiota and human host as well as among microbial clusters. A deeper characterization of bacterial population at genus level, for instance, is fundamental to reveal novel taxa as occurred for *Barnesiella* spp., *Dorea* spp. and *Desulfovibrio* spp., which was detected only few years ago [40]. Furthermore, it is important also consider those microorganisms which are present in low-abundance within bacterial communities as they may exert a pivotal role on the host's health; *P. gingivalis* and *C. difficile*, for instance, albeit scanty, can disrupt host-microbial homeostasis triggering inflammatory diseases [41], [42].

Therefore, since the evaluation of all deepest bacterial taxonomic levels may be very informative, we applied this analysis to investigate the effects of different potential probiotic strains on the human gut microbiota evaluating also microbial biodiversity between samples (Manuscript 3 and Manuscript 4). Studying the biodiversity of human microbiota has an enormous range of benefits since bacterial biodiversity has a strong impact on host's health and also contributes to clarify results and compare different datasets. In all ecosystem, a high microbial biodiversity promotes the maintenance of a balanced environment [43]. Indeed, an ecosystem with a great microbial diversity is more resilient and adaptable to stress than one in which the range of species is limited [44]. In our works, we evaluated the α-diversity since this allows us to understand how the bacterial community was differently composed, in term of richness and evenness, within each group analyzed. In literature, similar investigations have highlighted how same body areas differed extremely in both microbial composition and ecological organization. For example, vaginal bacterial communities are emerging as widely subjected to structural and temporal fluctuations despite they were described having the lowest alpha diversity [45]. Moreover, even if some bacterial genera are shared by most of individuals and represent a larger portion of particular body site communities, there are other specific taxa that are highly variable and never shared between individuals and different body areas. This aspect distinguishes in particular the oral microbiota as, even if it seems to be similar between different subjects, it changes mostly between the different oral surface within each individual (e.g., mucosa, saliva, and plaque) [46]. However, results obtained from bacterial abundances and biodiversity analysis alone are not sufficient to give a complete description of the microbiota population. For this reasons, they should be integrated with other analytic methods able to highlight potential interactions among microorganisms.

In our laboratory, we applied the Auto Contractive Map (Auto CM), a methodology based on an unsupervised Artificial Neural Network (ANN) architecture. We used this tool to investigate the composition of human breast milk microbiota (Manuscript 5). In addition, we performed a hierarchical cluster and principal components analysis, using in the present study as traditional benchmarking methods. From these approaches, we obtained extremely complex results which did not allow defining and recognizing which specific microbes could play a pivotal role within the ecological community. Conversely, the mathematical model of Auto CM highlighted the main bacteria that are potentially involved in homeostasis of both colostrum and mature microbiota.

In particular, the Auto CM algorithm constructs a bacterial network based on Minimum Spanning Tree theory that allowed us to underline the natural links among variables. Although MST-based clustering could appear formally equivalent to dendrograms produced by hierarchical clustering under certain conditions, visually they are extremely different. Moreover, both hierarchical clustering and PCA were obtained calculating eigenvectors of the correlation matrix which is a measure of similarity. Differently, the Auto CM exploited Kruskal's algorithm to arrange the arcs according to ascending cost order and subsequently analyzing them individually; the result is a graph with weighted arcs undirected. Comparing the four topology networks (Italian colostrum, Italian mature milk, Burundian colostrum and Burundian mature milk, respectively) we showed that the human milk microbiota is characterized by bacterial relationships having a dynamic nature, as they change within the same population during the transition from colostrum to mature milk. In addition, our study highlighted the impact that lifestyle and dietary habits could have in determining a different composition of the human microbiota. Although to date the real meaning of "bacteria switch" in the microbial networks is not completely clear, our results support the idea that Auto-CM may be a promising approach to better understand the complexity of human-microbes mutualism. In particular, the Auto CM may be a start point to characterize the framework of bacterial networks since it shows only the potential interactions between microorganisms, without giving any information about their biological meaning in the microbial population. Moreover, even if the application of Auto CM is undoubtedly a very interesting way to display and understand data, it is a not simple approach and it requires always first another type of analysis such as the manual filtering of the main data in according with the microbial relative abundance. For this reason, our aim is to acquire more computational skills with free tools available on the web, such as R and Cytoscape, two open source software which offer several tools for data mining and analysis. Indeed, the development of a Metagenomic Systems Biology is underway. Numerous mathematical and computational models besides those above mentioned, are already freely available for investigating the microbiome with a focus on network-based analyses [44, 45, 46]. These tools are aimed to characterize the assembly, organization, and activity of microbiome; however, they often require specific professionals, bioinformatics, or properly trained personnel who have extensive knowledge of programming and processing datasets.

Therefore, the lack of standard methodologies, but also of skills shared between researches and bioinformatics, represents the major problem that today still restricts the breakthrough of microbiome research applied to the clinical medicine. Nonetheless, the use of open source software is preferable since these methods have often additional plugin useful to define in details the analysis of the data (eg. gene annotations), but also to customize them according to standardized pipelines. In addition, differently from the Auto CM, open source software enable simultaneous analysis thus ensuring the honesty of metagenomics data.
Extensive efforts have been undertaken by The International Human Microbiome Standards (IHMS) project to establish standard operating procedures (SOPs) for both investigate the microbiome and evaluate the impact of a specific therapy or tool [50]. However, to date these SOPs were validated only on stool samples which represent a small fraction of biological samples available for research and clinical studies, such as bones and synovial and pleural fluids. In addition, another challenge that fascinates the scientific community is represented by the metatranscriptomics and metaproteomics, which widen the study of gene expression, considering also the biological and functional role of microbial communities. Anyway, the efforts of these survey methods, as well as of metagenomics ones, should be focused towards the characterization of a normal microbioma which is essential for understanding the role of bacterial populations in human diseases and that it is still absent in the scientific community. Indeed, even if association studies between gut microbiome and diseases have already highlighted several important interactions between host and intestinal microbes, much work is still required to discover and validate different microbial markers involved in human diseases. Therefore, the human microbiome research is undoubtedly a field of great interest that may improve our knowledge about several aspects of human health.

In conclusion, this PhD study allowed us to underline the main criteria that should be consider to lead the study of human microbiome from the bench to patients with clinical and diagnostic applications. First of all, a wide stratification of individuals belonging to the study population is absolutely necessary. Also the application of strict inclusion and exclusion criteria considering the genetic and lifestyle of both cohort and single individual is of fundamental importance. Moreover, standard experimental and analytical protocols, as well as datasets standardization procedures, should be adopted in order to reduce samples heterogeneity and ensure the reproducibility of

results. A collection of detailed metadata may allow a more specific and integrated analysis of dataset which, combined to system biology approach, based mainly on the study of microbial networks, may give a complete overview of the framework investigated. Subsequently, these analyses can be enriched applying metatranscriptomics and metaproteomics approaches.

Finally, the application of aforementioned workflows might lead to a potential noninvasive target-microbial diagnosis, based on the identification of potential bacterial markers that may be associated to different pathological states. Indeed, metagenomics is improving our knowledge of the human microbiota and the main bacterial populations constituting it. However, little is known about the specific role played by each microorganism and their interactions with the host [51, 52]. In this context, both the metatranscriptomic and metabolomics may provide further insights on the numerous functions of the microbiota in host's health, highlighting especially its implication in the onset and/or progression of different human diseases.

Appendix

Figure 1. Human breastmilk microbiota dendrogram. The graph was obtained by the hierarchical cluster analysis.

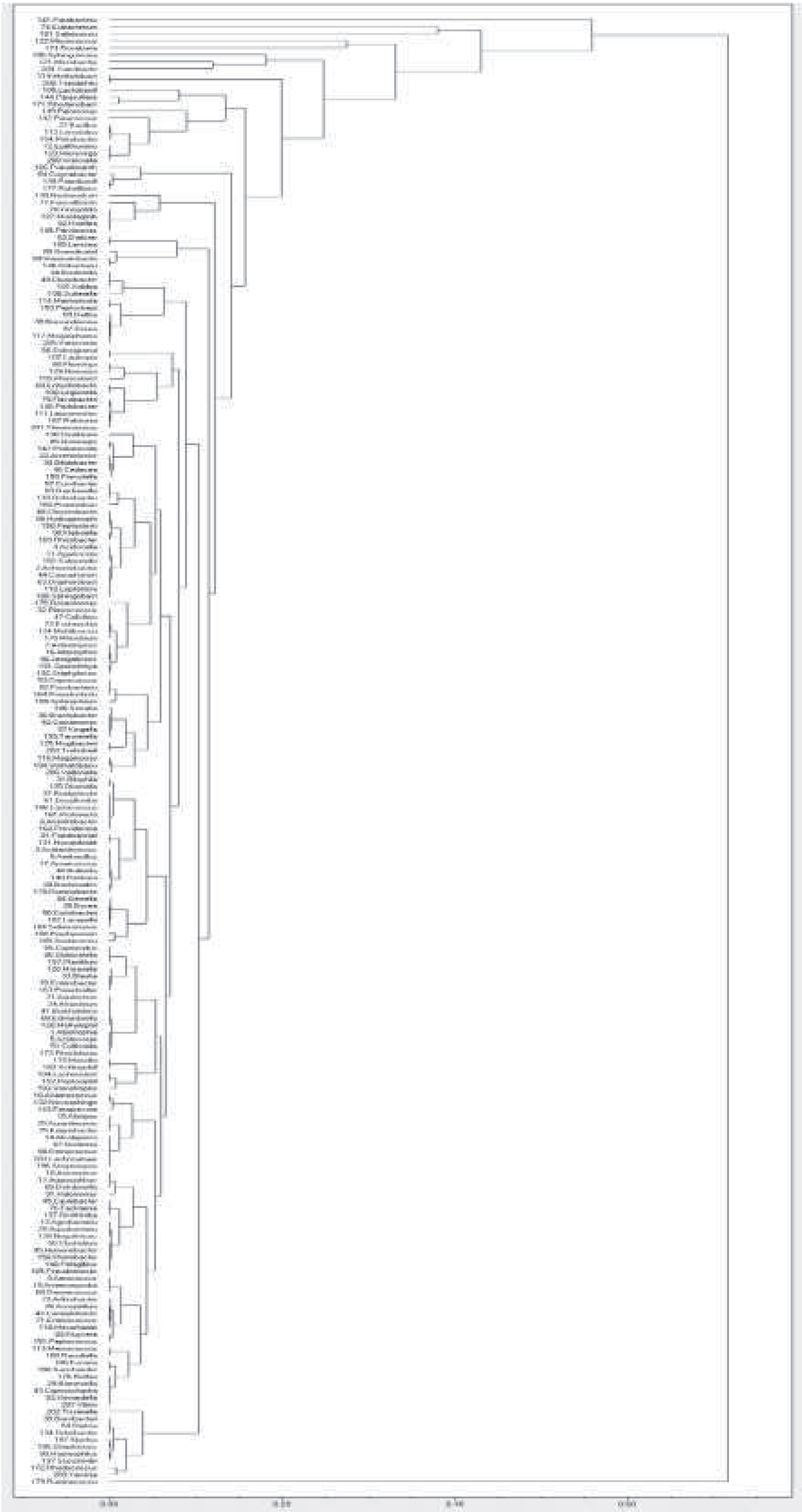
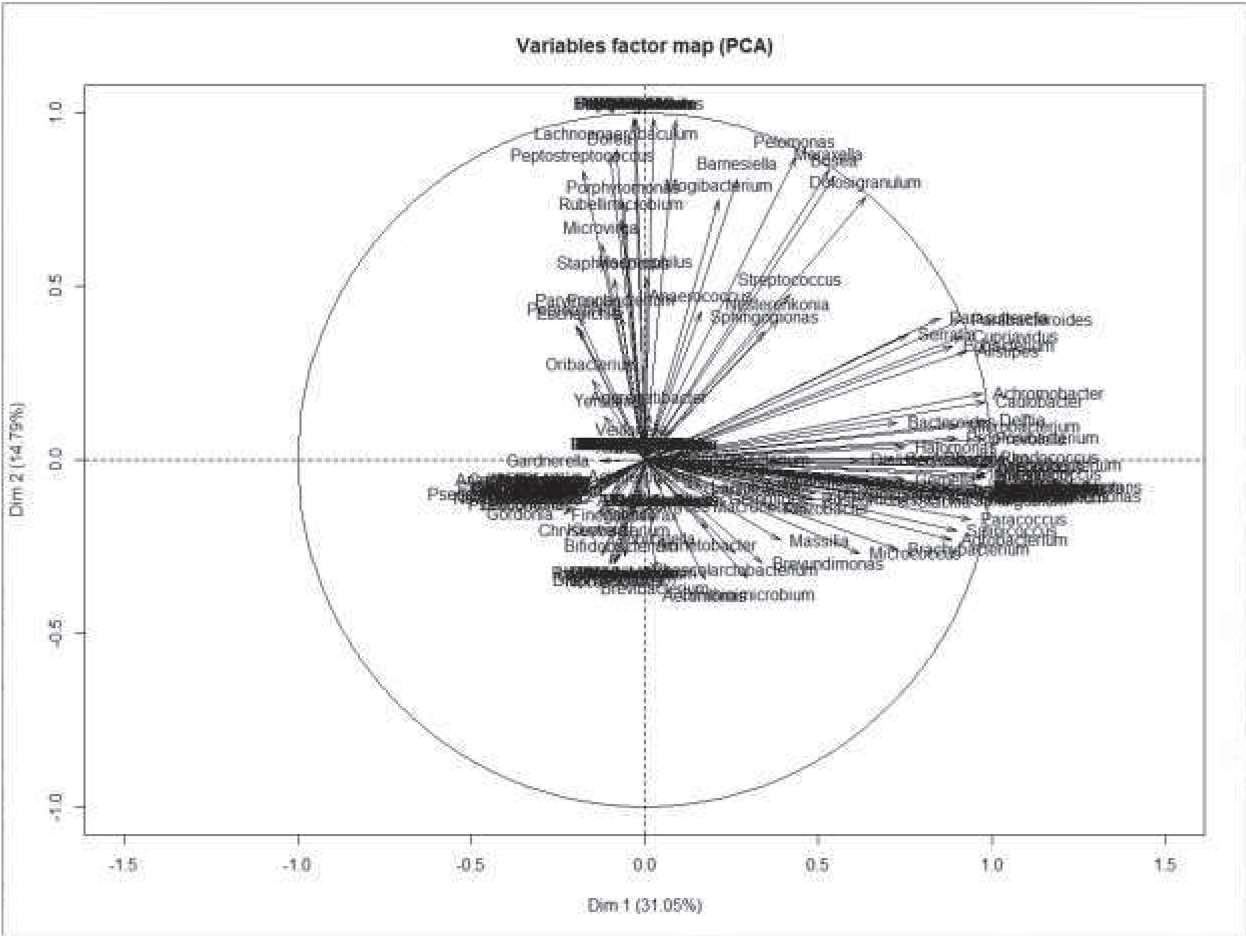


Figure 2. Human breastmilk microbiota PCA diagram. The graph was obtained by the PCA analysis.



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