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Bifidobacteria ecology of non-human primates: characterization of novel species with unexpected functionalities for probiotic applications and a co-evolutionary host-microbe analysis

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ABSTRACT

Bifidobacteria represent one of the main groups in the human and animal gastrointestinal tract (GIT) and are generally considered host-animal specific bacteria, with demonstrated health promoting properties. The basis of the close relationship between species of *Bifidobacterium* and their hosts is unknown, but it is thought to be due to peculiarities in the bifidobacterial cell-wall structures involved in intestinal epithelium adhesion, or to bifidobacterial ability to metabolize, in the intestine, specific substrates from the host diet. There is abundant information of bifidobacterial distribution in different animals distant on the evolutionary scale from humans, whereas few data are available on non-human primates bifidobacteria. Recently, a richness and diversity of bifidobacteria harboured by two species of New World monkeys, such as common marmoset (*Callithrix jacchus* L.) and red-handed tamarin (*Saguinus midas* L.) was observed and seem to introduce the existence of a storehouse of in the gut of primates, which should be deeply explored by the microbial ecologists. Novel bifidobacteria species, just considered probiotic for human health, could possess newly and improving unexpected functionality which may be studied for application in the environmental, pharmaceutical, agricultural or industrial fields. Taxonomists have been developed several fast and sensitive molecular approaches that provide detailed information about community structure of ecosystems in terms of richness, evenness and composition and can be used to compare different species present in environmental sample. These techniques are mainly based on the polymerase chain reaction (PCR), such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphisms (SSCPs), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphisms (T-RFLPs) and ribosomal intergenic spacer analysis (RISA). All his information could be offers new prospective to understand the evolutionary relationships between the gut microbiota and their hosts.

Beside the importance of characterization of bifidobacterial occurrence in microbial gut populations in new hosts also the detection of their origin could be a valuable information. When a definition of their origin, human or animal, is strictly a required, such as in "Microbial Source Tracking" studies as well as in probiotic selection, the RFLP-PCR of 16S rRNA gene sequences was described as a tool for speed up the discrimination of bifidobacteria.

The isolation and characterization of new bifidobacterial strains and species could play an important role also in the discovery of new features for their probiotic action. Furthermore, the effect of the probiotic strain on other members of the intestinal microbiota and importantly on the host are to be considered. Searching for EPSs-producing bacteria represents a new challenge in putative probiotic strains selection because EPSs possess beneficial health-promoting effects due to their crucial roles in adhesion mechanisms, control of pathogens, maintaining survival/viability of microorganism during technology food process/storage, and contributing to rheological properties of fermented foods. The ability to produce EPSs has been recognized in several bifidobacterial strains, but rapid tool for their screening, avoiding the time-consuming EPSs extraction and quantification, are currently unavailable. Recent studies have been highlighted the folate production from some bifidobacteria strains. Folate is a water-soluble B vitamin with important functions for the health, such as reduction of blood homocysteine levels, formation of red blood cells; folate is involved in the protein metabolism, cell growth and division and, in the first months of pregnancy, an adequate folate intake is necessary to prevent neural tube defects (NTD) and anencephaly. The folate produced by microorganism is natural folate which should thus be considered as a viable alternative to folic acid; indeed, compared to the synthetic folate, the microbial one does not cause "masking" of pernicious anaemia that occurs at high concentrations of folic acid

The main aim of this research project was the exploration of the bifidobacterial occurrence and diversity in different evolutionary primate hosts in order to i) improve the current knowledge about bifidobacteria distribution in non-human primates, ii) identify novel bifidobacteria species and iii) test their probiotic features, in particular focused the attention on the EPSs and folate production. Finally, based on our and literature explorations, the coevolution between bifidobacteria and their hosts was investigated.

The bifidobacterial diversity was explored in several non-human primate subjects to better understand the co-evolution between bifidobacteria and their primate hosts from different evolutionary time-scale. In detail, seventeen subjects selected from *Strepsirrhini* (*Lemuridae)*, *Eulemur macaco*, *Eulemur rubriventer*, *Hapalemur alaotrensis* and *Lemur catta*, and from *Simiiformes* (*Callithrichidae*), such as the New world monkeys *Callithrix jaccus*, *Pithecia pithecia*, *Saguinus oedipus* and *Saguinus imperator,* and from the Old world monkeys, *Chlorocebo aethiops* and *Macaca Sylvanus,* were investigated. Cultivable bifidobacteria were detected and isolated from twelve subjects out of seventeen different hosts monkey species: no bifidobacteria were isolated from the *Eulemur rubriventer*, *Hapalemur alaotrensis*, *Pithecia pithecia* and the old world monkeys.

Notable, the cluster analysis of the bifidobacterial isolates for the recognition of clones, using the program GelCompareII (AppleMath) in order to compare ERIC- and/or BOX- fingerprinting profiles, revealed a richness of unknown strains. Cultivable techniques allow us to isolated nineteen novel bifidobacterial species from common marmosets and tamarins together with five bifidobacterial species previously described. Actually, four novel species, *Bifidobacterium aesculapii, Bifidobacterium myosotis, Bifidobacterium tissieri, Bifidobacterium hapali* from common marmoset have been described and validated. At last in adult subjects of *Lemur catta* and *Eulemur macaco* the two novel species *Bifidobacterium lemurum* and *Bifidobacterium eulemuris* have been described.

Strains isolated from primates and tested for probiotics features, such as acid and bile tolerance, revealed that strains belonging to *B. aesculapii, B. myosotis* and the putative new species with type strain MRM_8.19 are the most resistant at extreme condition, showing the highest survival rates. The production of folate, verified on strains isolated from ring tailed lemur and common marmoset in a folate free medium (FFM), revealed presence of autotrophy for the vitamin only in strains from common marmoset, while no growth in FFM was recognized for strains from ring tailed lemur. *B. aescualpii* strains were tested for EPSs production after growth at different glucose and lactose concentration (1.5-2%). Outcomes suggest an increasing production at 1.5% of glucose (MRM_3.1 231.61 μg/ml), while very low amount was measured when lactose supplied the glucose as carbon source in the medium. Strains are unable to product significant EPSs when low fat cow milk was utilized as substrate, whereas high EPS production was recognized from strain MRM_4.6 (174.50 μg/ml) after soybean milk fermentation. The technological features measured rand elated to texture and aromatic analysis of fermented soybean and low-fat milks by *B. aesculapii* strains (MRM_4.8, MRM_5.13 and MRM_8.7) suggest the production of molecules characterizing yogurts, such as 2,3 butanedione.

The distribution of microbial communities in non-human primates from eight babies of common marmosets, golden faced saki and Barbary macaques and eleven adult subjects of ring-tail lemurs, black lemurs, red-bellied lemur, Alaotran bamboo lemur, Barbary macaques, grivet, cotton top-tamarin and emperor tamarin, was carried out by using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and rep-PCR. ARDRA results underlined the potential of the restriction analyses on the bifidobacteria 16S rRNA partial gene sequence, which seems able to distinguish harboured bifidobacteria at the species level. Getting an overview of the global community diversity, the rep-PCR fingerprinting analysis appears the best technique, even if no additional information about family, genus or species should be made. Results about bifidobacteria quantification suggest a low presence in evolutionary old primates, such as lemurs and old world monkey, compared to those in more evolved species, such as tamarins and common marmoset. Differently from bifidobacteria, the

amount of lactobacilli in faeces of common marmoset appeared not different in babies and adults, while, for Barbary macaques, lactobacilli and enterobacteria abundance showed the same trend of bifidobacteria increasing from baby to adult subjects.

Information from literature and all the isolations performed during these three years offer a based for co-evolution assumptions. The host-bifidobacteria coevolution of ring-tail lemur, black lemur, common marmoset, cotton top-tamarin and emperor tamarin and respectively related bifidobacteria was studied by different tree-based methods (TreeMap3, Jane4) and global-fit methods (PACo and ParaFit in CopyCat). The event-based methods did not find significant congruence between tree topologies, probably as a result of occasional host switching by the bifidobacteria and or due to possible failure to speciate events. The global-fit methods statistically support a global co-speciation between host-bifidobacteria, but not all the individual links in the system are significant.

Concluding, there is a wide storehouse of bifidobacteria in primates and these strains, after properly investigation regarding peculiar probiotic and/or technological features and accurate selection could be candidate for probiotic used mainly for the diary production.

CHAPTER 1

1. INTRODUCTION

The domain Bacteria is wide represented by the phylum *Actinobacteria* characterized by high G+C content (except for *Tropheryma whipplei*) and positive response at Gram staining (Lee and O'Sullivan, 2010). The phylum contains 6 orders, in which *Bifidobacteriales* can be divided in two families, *Bifidobacteriaceae* and *Incertae* (Biavati and Mattarelli, 2012). In the family *Bifidobacteriaceae,* all microorganisms are non-motile, non-endospore forming and usually strictly anaerobic, although sensitivity to oxygen is different among different species and genera (Mattarelli et al., 2014). The family includes 9 genera: *Bifidobacterium*, the most represented (50 species), *Alloscardovia*, *Aeriscardovia*, *Metascardovia*, *Parascardovia*, *Scardovia* (Biavati and Mattarelli, 2012), *Bombiscardovia* and *Pseudoscardovia* (Bunesova et al., 2013). The genus *Gardnerella,* which is Gram-variable, is also included in the family because both phylogenetically related to the other genera and possesses the key enzyme fructose-6-phosphate phosphoketolase (Mattarelli et al., 2014).

1.1. GENUS *Bifidobacterium*

Bifidobacterium genus is one of the most common bacteria intestinal group; it includes non-spore-forming and non-motile rods bacteria displaying different shapes with a typical bifurcated "bifid" morphology. Bifidobacteria are strict anaerobes, but some species could tolerate microaerophilic condition. Hexoses are fermented by an exclusive pathway whose the key enzyme is the fructose-6-phosphate phosphoketolase, with acetic and lactic acid being the main end products. The isolation habits include cavity and gastrointestinal tract of animals, and some species are isolated from human milk, but can also be present in sewage.

1.1.1. CLASSIFICATION AND ECOLOGY

Bifidobacteria were firstly discovered by Henry Tissier (1899) in breast infant faeces (Biavati and Mattarelli, 2012). Members of the genus *Bifidobacterium* dominate the indigenous microbiota of infants and as humans age increases bifidobacteria become one of the most abundant bacterial group following the genera *Bacteroides* and *Eubacterium* (Nicholson et al., 2012). The prominent species present in the human gut were *Bifidobacterium longum, Bifidobacterium pseudolongum, Bifidobacterium animalis* subsp. *lactis, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium pseudocatenulatum* and *Bifidobacterium breve* (Turroni et al., 2009). However an ecological survey of bifidobacterial populations associated with animal faeces revealed a broad distribution of bifidobacteria in the gut of a wide variety of animals, such as birds, ungulates, lagomorphs and rodents (Lamendella et al., 2008).

Microorganisms belonging to the genus *Bifidobacterium* are generally considered host-animal-specific and can be separated as "human group", "animal group" and others (Ventura et al., 2003).

Bifidobacteria showed a characteristic Y shape, even if cells morphology may differ between strains from short, regular, thin rods with pointed ends, to coccoidal regular cells, to long cells with a large variety of branching, and cells can occur both in singly or in chains (Mattarelli et al., 2014).

1.1.2. PHYSIOLOGY

As explained above, bifidobacteria are Gram-positive, non-motile, non-spore forming, generally strictly anaerobic bacteria, even if their sensitivity to oxygen may change accordingly to the species.

Optimal temperature growth conditions range between 37 and 41°C; extreme values where not growth is observed are below 20°C and above 46°C (Biavati et al., 2000). Gavini *et al.* (1991) proposed a discrimination between animal and human strains when they showed the growth at 45°C capability, with the exception of *Bifidobacterium thermacidophilum*, which is able to growth at 49,5°C (Dong et al., 2000).

Members of this genus are tolerant to acidity and their optimum pH range from 6,5 to 7,0; no growth is generally observed at pH values under 4,0 and over 8,5.

Bifidobacterium spp. possess a characteristic hexose catabolism, with the fructose-6-phosphate phosphoketolas is the key enzyme. This enzyme split the hexose phosphate to erythrose-4-phosphate and acetyl phosphate (Scardovi and Trovatelli, 1969). Through following actions of transaldolase and transketolase, tetrose and hexose phosphates were converted in pentose phosphates that, via the usual 2-3 cleavage, give rise to lactic acid and acetic acid in a theoretical (fermentation balance is influenced by formation of formic acid and ethanol, and could vary between species) final ratio of 1.0:1.5 (Biavati et al., 2000).

1.2. BIFIDOBACTERIA IN THE GUT OF HUMAN

The human gut microbiota represents a complex ecosystem colonized by a great number of bacterial cells, approximately 10^{12} per gram of feces (Kurokawa et al., 2007) and up to 10^{11}–10^{12} total bacteria in the human intestinal tract (Palmer et al., 2007). These microorganisms could be classified into more than 50 genera and hundreds of species in both the Bacteria and the Archea domain. The most represented genera in adults are five and, in the order, are *Bacteroides, Eubacterium, Bifidobacterium, Ruminococcus*, and *Clostridium; Bifidobacterium* is the most dominant genera in infants (Kurokawa *et al.*, 2007).

Concerning the distribution of *Bifidobacterium* spp. in the gut of infant and human adults, the species normally found are *Bifidobacterium adolescentis*, *Bifidobacterium angulatum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium infantis, Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum.*

Matsuki *et al.*, in a work published in the 2004, developed a method for PCR detection and distribution analysis of human intestinal bifidobacteria by combining real-time PCR using *Bifidobacterium* genus- and species-specific primers. The results obtained from this analysis are summarized by Kurokawa *et al.* (2007) in Table 1 and revealed that the population of bifidobacteria per gram of feces (average ± standard deviation) is about 9,4±0,7 $\log_{10}$. The most abundant is the *B. adolescentis* group, which is present in a relatively larger population (9,1±0,9 $\log_{10}$) and followed by the *B. catenulatum* group (8,9±0,8 $\log_{10}$) even if this group seems to be less common than *B. longum*. *B. longum* group is the most commonly detectable, but it is not the predominant species, exhibited a

populations ranging around 8,1±0,7 $\log_{10}$ that is generally smaller than those of the *B. adolescentis* and the *B. catenulatum* group.

Other studies reported *B. breve* and *B. longum* subsp. *infantis* as the major typical species of the intestinal tract of infants (Benno et al., 1984; Biavati et al., 1984).

Species	Genus *Bifidobacterium*	*B. adolescentis*	*B. angulatum*	*B. bifidum*	*B. breve*	*B. catenulatum*	*B. longum*	*B. infantis*
No. positive (%)	46 (100)	38 (83)	5 (11)	13 (28)	8 (17)	41 (89)	44 (96)	2 (4.3)
Mean ± s.d.	9.4 ± 0.7	9.1 ± 0.9	6.6 ± 0.2	8.3 ± 0.8	7.3 ± 0.7	8.9 ± 0.8	8.1 ± 0.7	6.9 ± 0.7
Range in positive subjects	[6.9; 10.6]	[7.4; 10.6]	[6.3; 6.9]	[6.8; 9.4]	[6.4; 8.4]	[6.3; 10.2]	[6.4; 9.4]	[6.4; 7.3]

* Minimum detection threshold of the method used: 6 Log_{10} CFU/mL.

Table 1. Distribution of *Bifidobacterium* spp. in the intestinal flora of human adults evaluated by quantitative PCR. Quantification is expressed as $\log_{10}$ bifidobacteria/g of faeces measured by reaction with genus- or species-specific primer. (Kurokawa *et al.* (2007) adapted from Matsuki *et al.*, (2004)).

1.3. BIFIDOBACTERIA AS PROBIOTICS

In the last decades an increasing focus on the probiotic activity of several microorganisms has been reported; their consumption provides health benefits to animals and humans. The definition most commonly use for "probiotics" is based on a report stipulated by the ILSI Europe and the WHO, and recently update in a consensus joint report between the Food and Agriculture Organization (FAO), and the World Health Organization (WHO), which defined probiotics "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014).

The original observation of the positive role played by selected bacteria in our gut is ancient. Over 2.000 years ago, Hippocrates stated that "all disease begins in the gut" and his wisdom found scientific evidence in the last decades. In the 1907 the Russian Nobel Prize winner Eli Metchnikoff suggested that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (WHO, 2006).

In the same time, the French paediatrician, Henry Tissier, observed that children with diarrhoea had in their stools a low number of bacteria characterized by a peculiar, Y- shaped morphology, "bifid" bacteria, which, on the contrary, were abundant in healthy children; so he suggested that these bacteria could be administered to patients with diarrhoea to help restore a healthy gut flora (WHO, 2006).

Probiotic microorganisms are often lactic acid bacteria (LAB), mainly belonging to two genera, *Lactobacillus* and *Bifidobacterium.*

Probiotics may play a role in improving epithelial barrier function, secretion of inhibitory substances, such as bacteriocins and hydrogen peroxide, immunomodulation, inhibition of expression of virulence factors and competitive exclusion – possibly through colonization resistance (Rea et al., 2013). The effects of probiotics could be classified in three actions (Oelschlaeger, 2010).

1. Modulation of the host's defences. It is related to the prevention and therapy of infection disease, in the treatment of chronic inflammation of the digestive tract and for the eradication of neoplastic host cells.

2. Direct effect on commensal and/or pathogenic microorganisms. Important for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut.
3. Probiotic actions that affect microbial products, such as toxins, or host products, such as bile salts and food ingredients, and result in inactivation of toxins and detoxification of host and food components in the gut.

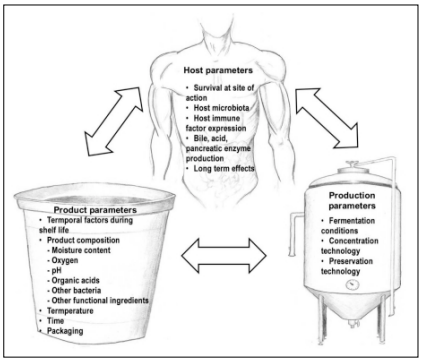

Figure 1. Factors (production parameters, product parameters and features of the host) that potentially impact probiotic viability and physiological state (Shane et al., 2010).

1.3.1. GUIDELINES FOR NOVEL PROBIOTICS

Since probiotic properties are strain related, the WHO, in 2006, defined that probiotics used for human consumption must be identified with methods of genetic typing, such as pulse field gel electrophoresis (PFGE). It also recommended that phenotypic tests may be done first, followed by genetic identification, using methods such as DNA/DNA hybridization, 16S RNA sequencing and other internationally recognized methods.

Generally well known requirements, to achieve successful outcome from probiotics therapy, are that the culture must be a common inhabitants of the human gut, non-pathogenic, able to colonize and also persist in high concentration, 10^7-10^9 CFU/ml of product (Vasiljevic and Shah, 2008). The selection criteria for lactic acid bacteria to be used as probiotics include to exert of beneficial effects on host, to remain at high cell counts and viable into the food products throughout its shelf-life, to survive through the git passage, adhere to the intestinal epithelium, to produce antimicrobial compounds and to posses antagonistic activity against pathogens, to stabilize the intestinal microbiota, have a human origin, to be stable at bile, acid, enzyme and oxygen exposition and to be safety, non-pathogenic, non-toxic, non-allergic, non-mutagenic and do not carry antibiotic resistance (Sharma et al., 2014).

In a joint of experts from FAO and WHO, in the 2002, a general agreement about key selection criteria for probiotics were established (see Table 2).

a) Safety Criteria

The first step is to assure the safety of the novel putative probiotic strain and it refers to the taxonomic identification, that gives information about its origin, habitat and physiology, and safety aspects, related to pathogenicity, infectivity and virulence factors such as antibiotic resistance. Although some authors in the past (Ouwehand et al., 1999) underlined the importance of the human origin, the expert of FAO and WHO (2002) advocated that probiotic action is more important than isolation source of the strain.

GENERAL	PROPERTY
SAFETY CRITERIA	• Origin
	• Pathogenicity and infectivity
	• Virulence factors—toxicity, metabolic activity and intrinsic properties, i.e., antibiotic resistance
TECHNOLOGICAL CRITERIA	• Genetically stable strains
	• Desired viability during processing and storage
	• Good sensory properties
	• Phage resistance
	• Large-scale production
FUNCTIONAL CRITERIA	• Tolerance to gastric acid and juices
	• Bile tolerance
	• Adhesion to mucosal surface
	• Validated and documented health effects
DESIRABLE PHYSIOLOGICAL CRITERIA	• Immunomodulation
	• Antagonistic activity towards gastrointestinal pathogens, i.e., *Helicobacter pylori*, *Candida albicans*
	• Cholesterol metabolism
	• Lactose metabolism
	• Antimutagenic and anticarcinogenic properties

Table 2. Main Criteria for selection of novel probiotic strains for commercial application (FAO/WHO, 2002).

In addition, to assure safety of novel probiotic strains for human consumption, Kumar *et al.* (2015) have been summarized some other important information to be investigated:

1. Genome announcement: it is recommending complete genome announcement and annotation. Functional annotation would help in predicting function.
2. Antibiotic resistance profile: all strains should be characterised for their antibiotic resistance potential and also the type of resistance. Conjugation studies could also be used to study transferability of antibiotic resistance.
3. Selection of proper in vivo model: there are growing numbers of studies, which are based on mouse and rat models. It is important to realise that these models do not provide the 'actual' gastrointestinal conditions of humans. However, preliminary testing could be essential for newly characterised strains or species.
4. Toxicological studies: some newly defined probiotic species are known to produce toxins. It should be scientifically assessed that the species or strain claimed for its probiotic properties does not produce any toxins.
5. Target population: target population should also be clearly defined, as a probiotic found to be effective in one population may have some adverse effect in another due to varied susceptibility to particular microbes. For example, application of probiotic for D-lactic acid production may lead to acidosis if used in infants.

b) Technological Criteria

Exploitation of beneficial health functions by probiotics is correlated to their ability in delivering the desired site in an active and viable form, even if Ouwehand *et al.*, in the 1999, noted funtional properties, such as immunomodulation, in non-viable cultures. About the viable recommended level, an agreement was not already reached and a suggested level range between 10^6

and 10^8 CFU/ml (Vasiljevic and Shah, 2008), considering declined in concentration during the passage through the gastrointestinal tract, or the processing and storage of the product that expose microorganisms to stress factors. During storage and food application, high acidity, substrate limitations, low water activity and low temperature may affect the survival and the activity of probiotics in the foodstuffs. Moreover, the survival depends also on the specific strain, on the interaction between probiotic and microorganisms natural presented in the system, production of peroxide due to bacterial metabolism and the final acidity of the environment (Vasiljevic and Shah, 2008). Other limitation factors are availability of nutrients, growth promoters and inhibitors, sugar concentrations, presence of dissolved oxygen and oxygen permeation through package, inoculation level, and fermentation time (Shah, 2000).

The presence of oxygen is crucial for microaerophilic and anaerobic strains, such as *Bifidobacterium* species. Strains belonging to this genus are affected by an incomplete reduction of oxygen to hydrogen peroxide due to the lack of an electron-transport chain. They are also devoid of catalase, which usually catalyses the conversion of hydrogen peroxide into water, so there is an accumulation of hydrogen peroxide in the intercellular space, which results in the death of the cells. Oxygen presences may be toxic for this group of probiotic strains.

The presence of antagonist microorganism in the foods or in the starter cultures may affect the growth/survival of probiotics, if there is a production of inhibitors compounds; on the other hand, an increase could be also observed if growth-promoting compounds are secreted. For example, Ishibashi & Shimamura (1993), have observed beneficial effects on the bifidobacteria growth when culture starters with photolytic or oxygen scavenging are presented.

Other technological aspects refer to the enhanced yields during the cultivation at the industrial scale and the improvement of the survival of probiotics during culture concentration and freeze drying (Vasiljevic & Shah, 2008).

c) Functional Criteria

In order to assure viability and persistence of probiotics in the food during the shelf life, during the passage through the acid condition of the stomach and to assure their resistance at the hydrolytic activities of enzymes and bile salts in the small intestine, probiotics strains have to be tested for their capability to survive under stress conditions. Commonly susceptibly to environmental conditions could be considered water activity, redox-potential (related to the presence of water), temperature and acidity (Vasiljevic and Shah, 2008). A final pH value below 4,4 leads to a decrease in the amount of probiotic cells in the foodstuffs. All these functional characteristics should be considered when probiotic strains are selected.

Strategies could be adopted to improve the persistence of probiotics during the storage, such as omission of antagonist starter culture strains, addition of probiotics after milk fermentation or addition of starter cultures after the probiotic fermentation.

In addition, the viability of probiotics during both the storage and the passage through the stomach, could be improved adding appropriate prebiotics, which are defined by FAO as "nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" (Pineiro et al., 2008). The benefit showed is linked to effects on the metabolic activity of probiotics, but the bacterial response is highly strain-specific (Vasiljevic and Shah, 2008).

Other functional characteristics are linked to health effects for the host associated with the consumption of probiotics, which may be influenced by the delivery matrix composition (Vasiljevic and Shah, 2008). Moreover, the probiotic activity during the fermentation could positively or negatively affect the nutritional and physiological value of the final products. Positive actions are related to photolytic activity showed by some strains, required for their rapid growth in the milk.

d) Desirable Physiological Criteria

When a putative new probiotic strain would be selected investigation of other desirable physiological characteristics should be performed. Adaptive response to macro- micronutrients availability, changing in the genetic expression, and in the enzymatic activity as consequence, production of EPS and biofilm formation represent only few examples of microbial physiology responses that could be measured.

The physiology activity of probiotics is important because related to health benefits for the host. Commonly health effects are strain specific and refer to alleviation of lactose intolerance, prevention and reduction of rotavirus and antibiotic associated diarrhoea or could potentially refer to treatment and prevention of allergy, reduction of risk associated with mutagenicity and carcinogenicity, hypocholesterolemic effect, inhibition of *Helicobacter pylori* and other intestinal pathogens, prevention of inflammatory bowel disease and stimulation of immune system (Vasiljevic and Shah, 2008).

1.3.2. MAIN PROBIOTICS TRAITS

Proper *in vitro* studies, followed by *in vitro* trials, are required to establish the potential health benefits of probiotic strains. Tests such as acid and bile tolerance, antimicrobial production and adherence ability to human intestinal cells should be performed depending on the proposed health benefit (Collins et al., 1998).

If the prevention of pathogen infection by a given probiotic is suggested, a clinical study is necessary to verify the exposure to the specific pathogen, preventive study, or the infecting microorganism is that specific pathogen, treatment study (WHO, 2006).

Commonly, when a new probiotic is described the main important features defined should be: a) absence of haemolytic activity, b) acid and bile tolerance, c) inhibition of pathogens, d) absence of antibiotic resistance, e) auto- and co-aggregation capability, f) coexistence test, and g) EPS and folate production; other traits could be also described for establish health effect of the strain, such as cholesterol removal, cell surface hydrophobicity and epithelial cells adherence assay (Bao et al., 2010; Guo et al., 2010; Tulumoglu et al., 2013).

a) Haemolytic Activity

Haemolysis refers to the ability of certain microorganisms to breakdown red blood cells. Normally, it is checked spreading tested cultures onto blood agar plates and verifying if they are able or not to induce haemolysis after growth at their optimal condition.

The haemolytic activity could be classified in 3 different types, (i) alpha- (α-), (ii) beta- (β-) and (iii) gamma- (γ-) haemolysis.

(i) α-haemolysis (or green or incomplete haemolysis), when a dark and greenish agar colour appears under the colony. It is a partial haemolysis caused by the hydrogen peroxide produced by microorganism that oxidise haemoglobin to methemoglobin (green). *Streptococcus pneumoniae* and a group of oral streptococci, such as *Streptococcus viridans*, belong to this type.

(ii) β-haemolysis (or complete haemolysis), when there is a complete lysis of red cells and, around and under the colonies a yellow and transparent halo could be observed. It is caused by the streptolysin, an exotoxin produced by microorganisms. This enzyme could be secreted in two forms by different group of bacteria, streptolysin O (SLO), an oxygen-sensitive cytotoxin that interacts with cholesterol in the membrane of eukaryotic cells, and streptolysin S (SLS) that

affects immune cells, including poly-morphonuclear leukocytes and lymphocytes, and is thought to prevent the host immune system from clearing infection.

(iii) γ-haemolysis (non haemolysis), when the colour of blood agar plates remains unchangeable because microorganisms are non haemolytic, such as *Enterococcus faecalis*.

b) Acid, Bile and Transit Tolerance

Potentially probiotic strains have to show surviving capability to passage through the gastrointestinal tract. At this purpose their acid, bile and gastrointestinal juice tolerance should be evaluated.

The low pH resistance is important to assure the viability of the probiotics during the passage through the stomach, where the pH value range between 2,5 and 3,5 (Holzapfel et al., 1998), but also to guarantee the microbial survival in the acid diary foods, such as yoghurt and fermented milks.

The capability to survive to bile exposure is an important requisite because bile secreted in the small intestine could destroy the cell membranes affecting the cell permeability and viability and altering the interaction between the membrane and the environment (Boke et al., 2010). A concentration of 0.15-0.3 % of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Boke et al., 2010).

c) Inhibition of Pathogens

The mechanism of action in preventing gastrointestinal infection of most probiotics is currently poorly understood. Probably it could be correlated to the bacteriocin-producing capability of certain strain. Dobson *et al.* (2012) proposed three possible mechanisms for the probiotic effect of bacteriocin production *in vivo*. The bacteriocin (i) acts as colonizing peptides by successfully allowing the probiotic strain to compete with the resident flora, (ii) kills peptides, resulting in elimination of the pathogen, or (iii) acts as signalling peptides through recruitment of other bacteria in the gut or the immune system to fight and eliminate the infectious organism. Bacteriocins produced by a probiotic may therefore function by facilitating the introduction of the strain into an established niche, directly inhibiting the invasion of competitors or pathogens into an established community, or modulating the composition of the microbiota and the host immune system (Rea et al., 2013).

The inhibition of pathogens is additionally related to the competition for limiting resources (such as iron), anti-adhesive and anti-invasive effects on the epithelial cells, and inhibition of toxins expression in pathogens.

The antimicrobial activity of probiotics is usually evaluated on a representative group of intestinal pathogens, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans* (Tulumoglu et al., 2013).

d) Antibiotic Resistance

In modern human and veterinary medicine, antibiotics are widely used representing the main defence against bacterial infections. Therefore, there are critical points in their usage mainly related to the selective pressure and the subsequent development of resistance mechanism in bacteria, probiotics, as well as the potential spread of resistance genes to pathogenic or commensal bacteria.

Ensuring the safety of probiotics for human and animals applications is essential. Probiotics and starter cultures might contain naturally occurring antibiotic resistance genes thus it is important to define the potential transmission of antibiotic resistance genes to unrelated pathogenic or potentially pathogenic bacteria in the gut. Indeed, bacterial strains harbouring transferable antibiotic

resistance genes should not be used in animal feeds, fermented and probiotic foods for human use (EFSA, 2008).

In the 2008, the European Food Safety Authority (EFSA) established the Qualified Presumption of Safety (QPS) approach, which defined that the nature of any antibiotic resistance determinant present in a candidate microorganism should be determined prior to approval for QPS status. The EFSA gives protocols to identify the Minimum Inhibitory Concentration (MIC) of the most relevant antimicrobials for each strain used as a probiotic organism, food or feed additives.

e) Auto- and Co-Aggregation Capability

The term aggregation refers to the process of reversible accumulation of cells. Two types of aggregation could be identified, (i) auto-aggregation, if the process involves bacteria which belong to the same strain, and (ii) co-aggregation, as result of cell-to-cell recognition between two different bacterial strains.

In probiotics, auto-aggregation has been correlated with adhesion to intestinal epithelial cells, known to be a prerequisite for colonization and enhanced persistence in the gastrointestinal system, while the co-aggregation of bacteria may form a barrier that prevents colonization by pathogens (Kos et al., 2003).

f) New Features: EPSs and Folate Production

Several authors have just highlighted the production of EPSs from many food grade microorganisms, such as LAB and propionibacteria (Cerning, 1995), but also from bifidobacteria strains (Andaloussi and Talbaoui, 1995; Liu et al., 2014; Roberts et al., 1995; Shaun et al., 2011)

The production of EPSs from microorganisms seem relate to cells defence and adhesion mechanisms. In the environment, the presence of EPSs protects cells from desiccation, osmotic stress, phagocytosis and phage attack, antibiotics or toxin compounds and predation from protozoans, moreover provides the cells ability to adhere to solid surface and to produce biofilm and cellular recognition (De Vuyst and Degeest, 1999).

In addition, several literature studies have described how some microbial polysaccharides may contribute to human health, either as non-digestible food fraction (Gibson and Roberfroid, 1995) or because of their antitumoral, antiulcer, immunomodulating or cholesterol-lowering activity (Welman and Maddox, 2003). Concluding, microbial EPSs have potential for development and exploitation as functional food ingredients with both health and economic benefits.

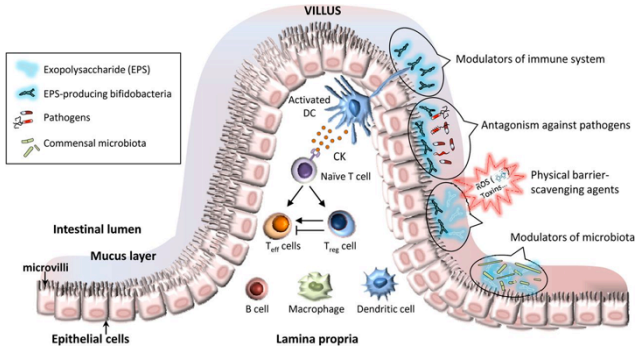

Figure 2. Beneficial activities potentially attributed to some EPSs synthesized by *Bifidobacterium*. CK, cytokines; DC, dendritic cell; Teff cells, lymphocyte T effector cells; Treg cell, lymphocyte T regulatory cell; B cell, lymphocyte B cell; ROS, reactive oxygen species (Hidalgo-Cantabrana et al., 2014).

Several authors recorded the production of folate by bifidobacteria (Crittenden et al., 2003; D'Aimmo et al., 2012a; Holasova et al., 2004; LeBlanc et al., 2007; Lin and Young, 2000).

A proper folate producing strains selection may represent a novel strategy for the development of novel functional foods with increased nutritional levels (LeBlanc et al., 2007). Indeed, natural folates, such as tetrahydrofolates produced by microorganism, do not cause "masking" of pernicious anemia that occurs at high concentrations of folic acid and should thus be considered as a viable alternative to folic acid fortification programs (Scott, 1999).

CHAPTER 2

2. PROKARYOTE SYSTEMATIC

The prokaryote systematic depends on the characterization of strain. To characterize a bacterium, several identification methodologies have been developed over the past 100 years, such as phenotypic analysis (peptidoglycan structure, fatty acid analysis, growth conditions, metabolism), genetic analysis (DDH, DNA profiling, DNA sequencing, GC ratio) and phylogenetic analysis (16S-based phylogeny, phylogeny based on other genes, multi-gene sequence analysis, whole-genome sequence based analysis).

2.1. MICROORGANISM CHARACTERIZATION, IDENTIFICATION AND DESCRIPTION

Several tests are needed to determinate whether a new isolated strain may be identified as a member of an existing or novel taxon and if a strain seems to be a novel one, it should be characterized as comprehensively as possible; and it should be allocated to a species and/or subspecies (Tindall et al., 2010). In the 2010, Tindall *et al.* published outlines to delineate and to show which methodologies are to be used for a clear characterization of a strain.

2.1.1. GENETIC-BASED METHODS

Since 1950s, the development of new genetic models, able to clarify the structure of DNA and deciphering the genetic code, has been chancing the prokaryote taxonomy. DNA G+C content value (De Ley, 1968), nucleic acid hybridization (DNA-DNA and RNA-DNA), analysis of the 16S rRNA gene (Fox et al., 1977), reverse transcriptase-sequencing (Lane et al., 1988; Sanger et al., 1977) and PCR-based gene sequencing (Saiki et al., 1988) analysis have been considered useful tools to investigate the prokaryotes taxonomy and evolution. Nonetheless, in the future of the microbial taxonomy is the whole genome sequence analysis of a microbial strain will represent an important step.

Nowadays, the use of information about 16S rRNA complete gene sequences homology plays a crucial role in novel taxa recognition and it represents a first indication, if a gene sequences similarity value less then 98,65% is retrieved (Kim et al., 2014), that a strain could be referred to a novel one. However, care should be taken when this tool is used. If similarity values are above that cut-off, other molecular information are required to establish if the strain belong or not to a new species or genera when the value is less then 95% (Tindall et al., 2010).

Sequence homology similarity values from other conserved genes (normally housekeeping genes), with a greater degree of resolution, could give help in resolving some issues. Multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST), based on sequences of about 7 housekeeping genes, are commonly used in order to better understand relationships among the species and their phylogenetic reconstruction.

2.1.2. PHENOTYPIC CHARACTERIZATION

Before the discovery of DNA and the development of molecular techniques, the identification, the characterization and classification of a microorganism were essentially based on its phenotypic features, by examination of morphological, physiological and biochemical properties.

Typical parameters describing the phenotype of a bacterium were cell shape, colony morphology, growth condition (pH, temperature, oxygen requirement), motility, formation o spores, biochemical profile. Other information are represented by the chemical cell composition (that referred to chemotaxonomy), such as fatty acid, polar lipid and respiratory lipoquinone composition, peptidoglycan cell wall amino acid composition, polyamine pattern.

2.2. RECOMMENDED MINIMAL STANDARDS

The importance of allocating a microorganism to a specific taxon lead to develop outline able to give at the researchers precise methods and criteria to collect detailed information required by new taxon description.

According to Recommendation 30b, before publication of the name and description of a new species, the examination and description should conform at least to the **Minimal Standards** (if available) required for the relevant taxon of bacteria (Stackebrandt et al., 2002).

Minimal Standards represented a useful document, a guideline, a list of characters and methods, that experts of each group of microorganism, joint in a Subcommittee, have compiled in order to enable taxonomists to correctly identify and allocate a strain to a specific taxon.

According to the Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology: *"Minimal characteristics should be provided and follow the guidelines set forth by various subcommittees of the ICSP. Where such guidelines do not exist, descriptions should follow guidelines for closely related taxa."* (Stackebrandt et al., 2002).

2.2.1. RECOMMENDED MINIMAL STANDARDS FOR DESCRIPTION OF NEW TAXA OF THE GENERA *BIFIDOBACTERIUM, LACTOBACILLUS* AND RELATED GENERA (MATTARELLI *ET AL.,* 2014)

The identification and the characterization of microorganisms are conventionally based on phenotypic and biochemical features, and also for *Bifidobacterium* spp., these features have to be considered (Scardovi and Trovatelli, 1969).

However, in the last decades a large amount of molecular techniques was introduced.

Most molecular tools for the bifidobacteria rapid identification are based on the 16S rRNA gene sequencing analysis, such as ARDRA (Ventura et al., 2001), DGGE (Temmerman et al., 2003) and species-specific primer (Matsuki et al., 1998). Other techniques, based on the repetitive genome

sequences amplification, are recently developed in front of the high similarity of the 16S rDNA of closely related *Bifidobacterium* spp. (Ventura et al., 2006), such as ERIC-PCR (Ventura et al., 2003), BOX-PCR (Masco et al., 2003), or GTG5 (De Vuyst et al., 2008) fingerprinting analysis. At the present, an innovative and alternative molecular tool based on the restriction endonuclease analysis of the PCR-amplified *hsp60* (*hsp60* PCR-RFLP), was developed for a rapid, reproducible and easy-to-handle identification of *Bifidobacterium* spp. at the species level (Baffoni et al., 2013); an update of the *hsp60* PCR-RFLP applied to bifidobacteria has been described in **PAPER 1**.

In 2014, **Minimal Standard Requirements for the description of novel species of the genera *Bifidobacterium, Lactobacillus* and related genera** were outlined by the Subcommittee on the Taxonomy of *Bifidobacteria*, *Lactobacillus* and related genera (Mattarelli *et al.*, 2014).

This document represents an instrument for taxonomists in order to correctly identify and allocate strains to a taxon, at genus and species level. It can be used not only at taxonomic purposes, but also for routine investigations for species and genera considered by the scope of the International Committee on the Systematics of Prokaryotes (ICSP).

Minimal standard requirements about ecology, phenotype and genotype, essential for description and differentiation of new taxa, have been defined and can be improved with optional additional characteristics.

a) Ecological Criteria

Ecological features of a microorganism refer to source and habitat of isolation. This description should be complete. For bifidobacteria is mainly significant being often used as supplements or starter cultures in foods or pharmaceutical preparations (Biavati and Mattarelli, 2012). Authors should highlighted possibility of transfer from primary to secondary habitats, or the addition in foodstuffs with strains isolated from other animals.

In latest years studies on the complex ecosystem of gut microbiota have gained important progress due to culture independent techniques, such as DGGE, pyrosequencing and metagenomics (Turroni et al., 2012). These are powerful tools able to describe the whole microbiota in isolated sample and to give an ecological overview of investigated habitat. However, when the aim of the work is to characterize single isolates, only culture dependent techniques can be used.

Several studies demonstrated the importance to isolate and identify novel *Bifidobacterium* strains from several animals, including humans, in order to clarify their distribution (Endo et al., 2012; Tsuchida et al., 2014). High number of studies has been targeted to human gut microbiota, but very few studies concern non-human primates.

b) Phenotypic Criteria

Phenotypic criteria generally refer to morphological, physiological, biochemical and nutritional characteristics.

Morphological main features are cell shape, which should described utilizing a photomicrograph; because morphology is closely dependent on growth conditions, a description of the culture parameters and medium composition should be also given. Bifidobacterial cells are typical for branch forming and it should be important that authors provide disposition and number of branches of the cells in different condition (agar or broth medium).

A complete description of colony morphology also required and refers to size, shape, colour, edge, elevation, surface, consistency and transparency of colony.

Cultures should be also examined for motility capability, behaviour of the cells in Gram-staining and endospore formation (bifidobacteria and lactobacilli are non-endospore forming bacteria).

Another important morphological feature is the fermentation pattern; microorganism of the *Bifidobacterium* genus, considered in the present thesis, indeed are characterized by strictly saccharoclastic activities and bifidobacteria should be tested for fermentation of cellobiose, melibiose, raffinose, mannitol, amygdalin, sucrose, galactose, lactose, maltose, mannose, salicin, trehalose, arabinose, aesculin, gluconate, melezitose, ribose, sorbitol, xylose, fructose, glycerol, rhamnose and starch. For bifidobacteria new taxon description it is essential evidencing the key action of the fructose-6-phosphate phosphoketolase on the catabolism of the fructose 6-phosphate.

Range and optimal growth conditions are to be investigated in term of temperature, pH, and demand in oxygen. Bifidobacteria are generally strictly anaerobic so they should be tested for the absence of catalase.

Additional phenotypic characteristics, such as antibiotic susceptibility and bacterocin typing, can be useful for the characterization of a new taxon, although they are not generally considered as part of the 'minimal standards'.

Characteristics able to define the phenotype of a new taxon are significant in the description of a new species, but, as well known, theses could be affected by cultural and test conditions. To overcome that issue, authors have to include in the description strains of relevant reference taxa together with the type strain of the type species of the genus (Mattarelli et al., 2014).

c) Genotypic Criteria

In the last decades, more attention has been given at identification techniques based on the nucleic acid composition. DNA-DNA hybridization and other genetic sequence-based methods are considered main tools for delineate relationships between taxa.

DNA base composition refers to the guanine and cytosine content (G+C mol%) and leads to a demarcation between high and low G+C content, as well as between Gram-staining positive and negative bacteria. The determination of the G+C percentage could be measured with different methods, such as enzymically hydrolysing DNA and subsequent quantification of the nucleosides by HPLC, complete genome sequencing, etc. Microorganism belonging to the genus *Bifidobacterium* can show a high range in G+C content, 10% within species of the genus (Mattarelli et al., 2014).

A powerful tool for the estimation of genetic relationships at the species level in prokaryotes is represented by **DNA–DNA hybridization (DDH)**. Though it is not always required, it is necessary when a value of 16S rRNA gene sequence similarity above 97% between a stain and its related is retrieved.

16S rRNA complete gene sequencing represents an easy to use and common marker in the phylogenetic relationship analysis. The large amount of sequences in public databases, free on-line available, represents an advantage in utilizing this methods for phylogenetic relationship reconstruction. It is able to give a first indication that a new species was found when a value of sequence similarity below 98,65% (Kim et al., 2014) between a strain and its closest neighbours is showed.

16S rRNA gene sequence similarity is not sufficient and **complementary phylogenetic markers** are required for a correct phylogenetic analysis. Set of multiple housekeeping genes, such as *hsp60, rpoB, dnaJ*, *dnaG, rpoC, purF, clpC*, *xfp* and non-encoding (intergenic) regions, such as 16S–23S internally transcribed spacer (ITS), are able to show a great degree of resolution. Zeigler (2003) has defined rules that help authors in the selection of genes for species discrimination: (i) genes should be present in one or few copies in most bacterial genomes; (ii) they should possess a higher rate of evolution when compared with rRNA genes; (iii) they should not easily recombine; (iv) they should possess enough variability to allow discrimination of species in a given genus.

The following additional genotypic characteristics that could be valuable for novel species or genus descriptions are recommended as supporting data when possible are: plasmid profiling, multilocus sequence typing (MLST), multiple locus variable number of tandem repeats analysis (MLVA) and genomic fingerprinting.

d) Chemotaxonomic Criteria

The most important chemotaxonomic feature for grouping and identifying members of bifidobacteria is the peptidoglycan structure. In the 1972, Schleifer & Kandler highlighted the taxonomic relevance of the nature of the di-amino acid and/or the interpeptide bridge of the cell-wall peptidoglycan.

Additional chemotaxonomic characteristics concern fatty acid analysis, distribution pattern of polar lipids, whole-cell protein profiling, electrophoretic mobility of enzymes and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS, may be valuable for novel species or genus descriptions and are recommended as supporting data whenever possible.

2.1. CASE OF STUDY 1. UPDTATING A RAPID TOOL FOR SPECIES IDENTIFICATION

Refers to **PAPER 1.**

Recently, an increasing interest concerning the probiotic potential of specific *Bifidobacterium* strains have been induced the exploration of uninvestigated habitats. An efficient and rapid tool for the identification of isolated strains is essential, and different molecular markers have been proposed. The high conserved *hsp60* gene is considered as an accurate tool for species identification and phylogenetical analysis within the genus *Bifidobacterium* (Jian et al., 2001). In the 2013, Baffoni *et al.* developed a new method based on the PCR-Restriction Fragment Length Polymorphism (RFLP) analysis of *hsp60* gene, using a unique enzyme, *HaeIII*, for a rapid and accurate identification of common species of bifidobacteria.

2.1.1. AIM OF THIS SECTION

Aim of this work was the updating of the previous work including new recently isolated and validate bifidobacteria. In addition to all the 25 species of *Bifidobacterium* genus and the subspecies belonging to *B. pseudolongum* and *B. animalis*, further 11 bifidobacterial species and *Bifidobacterium denticolens* and *Bifidobacterium inopinatum*, recently reclassified as separated related genera, *Parascardovia denticolens* and *Scardovia inopinata,* respectively, were processed using the technique described by Baffoni *et al.* (2013). The diagnostic dichotomous key proposed to faster the identification process has been kept up *including* all the additional species analyzed.

2.1.1. MATERIALS AND METHODS

Materials and methods are wide explained in both Baffoni *et al.* (2013) and **PAPER 1**.

Briefly, theoretical restriction profiles were obtained with an *in silico* analysis using *hsp60* sequences retrieved from the EMBL and GenBank nucleotide databases. Digestions with *HaeIII* was simulated by using Webcutter2.0 (http://rna.lundberg.gu.se/cutter2/). Nevertheless, the *hsp60* sequences of *B. stellenboschense* and *B. mongoliense*, which were not available, were amplified and cloned using InsTAclone PCR Cloning Kit (Fermentas) and then sequenced by Eurofins MWG Operon. Sequences were then submitted to GenBank sequence database. *In silico* profiles were confirmed by theoretical analysis. Type strains of each species used for this study were obtained from DSMZ (Deutsche Sammlung von Mikroorganism) and are listened in the Table 1, **PAPER 1**. DNA from overnight cultures, anaerobically grown on TPY broth for 24 hours at 37°C, was extracted using the method by Rossi *et al.* (2000). The initial PCR-RFLP method was slightly modified to improve the *hsp60* gene amplification, and 1 µl of DSMO was added in each 20 ml PCR reaction mixture. After digestion with *HaeIII* enzyme, theoretical restriction profiles have been obtained for all the species on 4-20% polyacrylamide gels (PAGE Gold Precast Gels, Lonza).

2.1.1. RESULTS AND DISCUSSIONS

Results and Discussions are clarified in **PAPER 1**.

We processed a total of 13 species and 11 of them showed a specific RFLP profiles, allowing a simple identification of the taxa under investigation. Indeed, three species presented distinctive high fragments, 537, 462 and 559 bp for *B. tsurumiense, B. callithricos* and *S. inopinata,* respectively. Profiles have been not observed in the previous work and not due to possible incomplete digestion of such long amplicon.

Due to absence of cutting sites for *HaeIII*, no RFLP profiles were generated from B. *psychraerophylum* and B. *actinocoloniforme,* and a single undigested amplicon of 590 bp were obtained. In order to correctly identify these 2 species, *hsp60* or 16S rRNA partial sequencing can be performed. This work allowed us to obtain 11 new distinctive RFLP profiles updating the previous number of species that may be distinguished with this technique. In addition, the rapid interpretation of the restriction profiles should be performed by the use of the update diagnostic dichotomous key, which has been kept up, including all the species analyzed.

Once again, the potential of the RFLP-PCR method based on the *hsp60* for a rapid and efficient identification of the 36 analyzed species of *Bifidobacteriaceae* is confirmed.

2.2. CASE OF STUDY 2. DESCRIPTION OF NOVEL SPECIES

Refers to **PAPERS 2, 3, 4, 5** and **DRAFTS 1**.

During the last three years, we explored the bifidobacterial occurrence in faeces of ten species of monkeys from different evolutionary time-scale, such as *Callithrix jaccus, Saguinus oedipus, Saguinus imperator, Pithecia pithecia, Chlorocebo aethiops, Macaca sylvanus, Lemur catta, Eulemur macaco*, *Eulemur rubriventris* and *Hapalemur alaotrensis*

After cluster analysis, to group isolated clones, and identification, by *hsp60* RFLP-PCR or 16S rRNA gene sequencing, the presence of frequent unknown strains belonging to the *Bifidobacterium* genus has been showed.

2.2.1. AIM OF THIS SECTION

Aim of this part of our research was the improvement of the ecological and diversity knowledge about bifidobacteria in the gut of primates.

After isolation and identification analysis, putative novel species were correctly validate from the phenotypic and genotypic point of view; the phylogenetic relationship with the other species in the genus *Bifidobacteria* were also reconstructed in accordance with the Minimal Standards (Mattarelli et al., 2014).

2.2.2. MATERIALS AND METHODS

Monkey Species	Monkey Group	Acronym	Number of subject	Age
Common Marmoset ***Callithrix jacchus***	New World Monkeys	MRM	8	Mix adults
Common Marmoset ***Callithrix jacchus***	New World Monkeys	MRM	4	Mix babies
Common Marmoset ***Callithrix jacchus***	New World Monkeys	MRM	4	Mother and babies
Common Marmoset ***Callithrix jacchus***	New World Monkeys	MRM	5	Individually babies
Emperor tamarin ***Saguinus imperator***	New World Monkeys	TRI	1	Individually adult
Cotton top tamarin ***Saguinus oedipus***	New World Monkeys	TRE	1	Individually adult
White-faced saki ***Pithecia pithecia***	New World Monkeys	SK	1	Individually adult
Grivet ***Chlorocebo aethiops***	Old World Monkeys	CRT	1	Individually adult
Barbary macaque ***Macaca sylvanus***	Old World Monkeys	BRT	3	Individually adults
Ring-tail lemur ***Lemur catta***	Lemurs	LMC	2	Individually adults
Black lemur ***Eulemur macaco***	Lemurs	LMM	2	Individually adults
Red-bellied lemur ***Eulemur rubriventris***	Lemurs	LMR	1	Individually adult
Lac Alaotra Bamboo lemur ***Hapalemur alaotrensis***	Lemurs	LMB	1	Individually adult

Table 3. List of sampled monkeys with information about scientific name, monkey group, laboratory acronym, number and kind (adult or baby) of subject.

Fresh fecal samples were individually collected from primates subjects of *Callithrix jacchus*, adult and baby individuals kept in Aptuit s.r.l., Verona, Italy, and from adult subjects of *Saguinus oedipus*,

Saguinus imperator Pithecia pithecia, Chlorocebo aethiops, Macaca sylvanus, Lemur catta, Eulemur macaco, *Eulemur rubriventris*, and *Hapalemur alaotrensis*, housed at Parco Natura Viva of Pastrengo, Verona, Italy Table 3.

All animals were weaned, free from intestinal infections and did not receive antibiotics or probiotics for two months before samples were collected. The diet of the babies consisted in pelleted feed (specifically for marmoset), fresh and dried fruit, bread and biscuits, eggs, gum arabic (or acacia gum) and milk and cranberry juice. The adult feed was divided in 2 meals, one in the morning with different kind of fruits and another with vegetables in the afternoon. Moreover, lemurs were provided with an environmental enrichment program to promote species-specific behaviors (Hosey et al., 2013) and, once a week animals received other foodstuffs including a mineral-vitamin supplement for primates, carbohydrates (legumes) and protein (eggs, live larvae of *Tenebrio molitor* or yogurt).

Materials and Methods are accurately described in **PAPER 1, 2, 3 and DRAFT 1, 2, 3**.

Summarizing, bifidobacteria were enumerated by plate counting and about 20-50 colonies were isolated in selective media, such as TPY added with mupirocin and TOS. Genomic DNA of pure cultures was extracted and, after grouping clone strains by cluster analysis, the *hsp60* RFLP-PCR or the 16S rRNA gene sequences of representatives were used for identification. If the gene homology was lower then the 98% with the species in the NCBI database, additional genetic analyses (*hsp60, rpoB, clpC, dnaJ, dnaG and rpoC* partial gene sequencing) were conducted for the phylogenetic collocation of the strains. Putative novel bifidobacterial species where described according to the **Recommended Minimal Standard** (Mattarelli et al., 2014).

2.2.3. RESULTS AND DISCUSSIONS

The isolations performed on primate fecal samples allowed us to find numerous putative novel species belonging to the *Bifidobacterium* genus. The describing analysis is an expensive and long cost-time work; therefore, currently, only eleven species have been taken into account. Six out of them are already validate, while for the other works are in progress.

Cultivable bifidobacteria were detected in subjects of 8 different host monkeys species. We isolated strains belonging to the *Bifidobacterium* genus from *Lemur catta*, *Eulemur macaco, Callithrix jaccus, Saguinus oedipus* and *Saguinus imperator*. Unfortunately, no success was achieved from the Lemuridae *Hapalemur alaotrensis* and the Old World Monkey *Chlorocebo aethiops* and *Macaca sylvanus*.

From five baby subjects of the common marmoset, the small exudivore monkey from the New World (*Callithrix jacchus*), six bifidobacterial strains with similar but peculiar morphology were first isolated and subsequently identified as the novel species *Bifidobacterium aesculapii* (**PAPER 2**). From the same subjects we were also able to isolate and identify other three novel species, viz. *Bifidobacterium tissieri, Bifidobacterium myosotis* and *Bifidobacterium hapali* (**PAPER 4**). The same subjects harboured also two bifidobacteria strains candidate as novel taxa but they are not already described.

Following these results, we investigated the occurrence of bifidobacteria in the faecal microbiota of a five years old subject of the ring tailed lemur (*Lemur catta*), which is one of the most well-known and recognizable species of lemurs but also the single type specimen of the genus Lemur. In this subject we observed cells of a bacterium with a peculiar morphology resembling a small-coiled snake or a little ring. A total of four isolates with this morphology were obtained from this one adult and were subsequently identified as a novel species and described as *Bifidobacterium lemurum* (**PAPER 3**).

At the same time, we had also the possibility to study two adult subjects of the black lemur (*Eulemur macaco*). The black lemur is one of 28 species of lemurs, from the family of Lemuridae, which are primitive relatives to monkeys and apes. The black lemur is unique among lemurs whit the feature that males and females have different colours. In these two subjects we were able to isolate the previously described species *Bifidobacterium lemurum* together with a novel species, which has been described as *Bifidobacterium eulemuris* (**PAPER 4**).

From Parco Natura Viva of Pastrengo, Verona, we obtained fresh fecal samples from other two New World Monkeys, belonging to the same family of common marmoset, *Callitrichidae,* such as the cotton top tamarin (*Saguinus oedipus*) and the emperor tamarin (*Saguinus imperator*). We isolated about 40-45 colonies from each animal. Cluster (BOX-PCR) and identification (sequencing of the 16S rRNA gene) analyses of isolates from the cotton top tamarin revealed 8 groups of novel bifidobacteria candidate species and three of them are currently under description as *Bifidobacterium aeriphilum*, *Bifidobacterium avesanii* and *Bifidobacterium ramosus* (**DRAFT 1**), while the other 5 will be described further.

PAPER 1

Stenico, V., **Michelini, S.**, Modesto, M., Baffoni, L., Mattarelli, P. & Biavati, B. (2014). Identification of *Bifidobacterium* spp. using *hsp60* PCR-RFLP analysis: An update. *Anaerobe* 26, 36–40. doi: 10.1016/j.anaerobe.2013.12.004.

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Note

Identification of *Bifidobacterium* spp. using *hsp60* PCR-RFLP analysis: An update

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ABSTRACT

A PCR-RFLP technique has been applied on 13 species of *Bifidobacterium* in order to update a previous study carried out by Baffoni et al. [1]. This method is based on the restriction endonuclease activity of *HaeIII* on the PCR-amplified *hsp60* partial gene sequence, and allows a rapid and efficient identification of *Bifidobacterium* spp. strains at species and subspecies level.

Members of the genus *Bifidobacterium* are high G+C Gram positive, obligate anaerobic, non-motile bacteria and represent an important constituent of human and animal gut microbiota [2]. The genus *Bifidobacterium* hosts 42 species, but this number will tend to grow. The increasing interest concerning the probiotic potential of specific *Bifidobacterium* strains induces to explore uninvestigated habitats, searching for new species. As an example of the wide unknown bifidobacterial biodiversity, five novel species were discovered during the study of common marmoset and red handed tamarin microbiota [3]. In this context an efficient identification of isolated strains is essential, and different molecular markers have been proposed to this aim. At present time, the highly conserved *hsp60* gene is considered as an accurate tool for species identification and phylogenetical analysis within the genus *Bifidobacterium* [4]. PCR-RFLP analysis of *hsp60* gene, the new method recently described by Baffoni et al. [1], allowed a rapid and accurate identification of common species of the genus *Bifidobacterium*. A single restriction enzyme (*HaeIII*), generated a RFLP profile specific for each species analyzed. In order to update the previous work, further 11 bifidobacterial species and *Bifidobacterium denticolens* and *B. inopinatum* (recently reclassified as *Parascardovia denticolens* and *Scardovia inopinata* respectively) were processed using the technique described in Baffoni et al. [1]. The type strains used in this work were obtained from DSMZ (Deutsche Sammlung von Mikroorganism) and are listed in Table 1. Cells were grown on TPY medium [5] under anaerobic conditions and incubated at 37 °C for 24/48 h. DNA was extracted using the method described in Rossi et al. [6]. PCR-RFLP method was slightly modified: 1 μl of DSMO was added in each 20 μl PCR reaction mixture, in order to improve the *hsp60* gene amplification. An *in silico* analysis was performed to obtain the theoretical restriction profiles, using the *hsp60* sequences retrieved from the EMBL and GenBank nucleotide databases and using Webcutter2.0 (http://rna.lundberg.gu.se/cutter2/). Nevertheless the *hsp60* sequences of *B. stellenboschense* and *B. mongoliense*, which were not available, were first amplified and cloned using InsTAclone PCR Cloning Kit (Fermentas) and then sequenced by Eurofins MWG Operon. Sequences were then submitted in GenBank sequence database and the accession numbers were listed in Table 2. Theoretical restriction profiles have been confirmed for all the species on 4–20% polyacrylamide gels (PAGEr® Gold Precast Gels, Lonza) (Fig. 1). 12 species showed specific RFLP profiles, allowing a simple identification of the strains under investigation. *B. tsurumiense*, *B. callithricos* and *S. inopinata* RFLP profiles presented distinctive high fragments (537, 462 and 559 bp respectively), never observed in the previous work and not due to possible incomplete digestion of such long amplicons [1].

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Table 1
List of species investigated.

Bifidobacterium species	International culture collection	Source
B. actinocoloniforme	DSM22766	Bumblebee digestive tract
B. bohemicum	DSM22767	Bumblebee digestive tract
B. reuteri	DSM23975	Feces of common marmoset
B. psychraerophilum	DSM22366	Pig cecum
B. bombi	DSM 19703	Bumblebee digestive tract
B. tsurumiense	DSM17777	Hamster, dental plaque
B. mongoliense	DSM 21395	Airag, Mongolian fermented beverage
B. saguini	DSM23967	Feces of tamarin
B. stellenboschense	DSM23968	Feces of tamarin
B. biavatii	DSM23969	Feces of tamarin
B. callithricos	DSM23973	Feces of common marmoset
Parascardovia denticolens	DSM 10105	Human dental caries
Scardovia inopinata	DSM 10107	Human dental caries

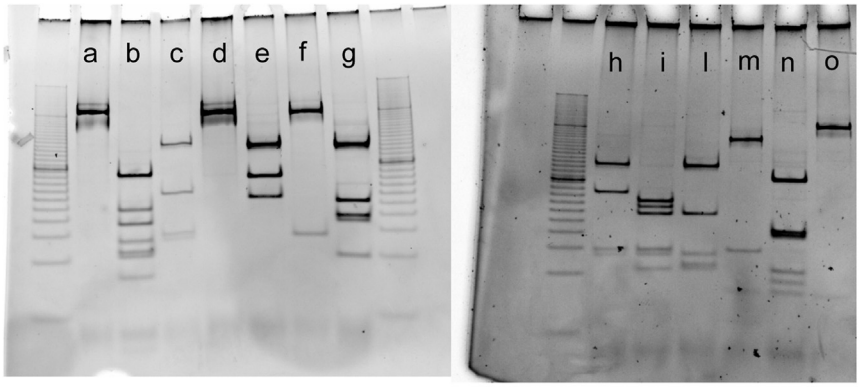
For *B. psychraerophylum* and *B. actinocoloniforme* no RFLP profiles were generated and a single undigested amplicon of 590 bp were obtained, due to absence of cutting sites for *HaeIII* (lanes a and d). In order to correctly identify these 2 species, *hsp60* partial sequencing can be performed. For most of the species tested in this work only type strains were available in the International Culture Collections, and for this reason the intraspecies profiles conservation were not evaluated.

With this work we obtained 11 new distinctive RFLP profiles (listed in Table 2), updating the previous number of species [1] that may be distinguished with this technique. For a faster interpretation of the restriction profiles, the diagnostic dichotomous key has been kept up, including all the species analyzed (see Table 3). This method confirms once again its discriminating power, allowing an efficient identification of 36 analyzed species of *Bifidobacteriaceae*.

Table 2
Expected fragment size obtained with *in silico* digestion of the *hsp60* gene sequences.

Bifidobacterium species	GenBank entry	Predicted fragment sizes
B. actinocoloniforme	GU223107	No sites
B. adolescentis	AF210319	31–36–81–103–339
B. angulatum	AF240568	42–54–59–139–296
B. animalis subsp. *animalis*	AY004273	17–53–86–97–114–223
B. animalis subsp. *lactis*	AY004282	71–86–96–114–223
B. asteroides	AF240570	30–38–75–97–109–242
B. biavatii	**AB674321**	**14–16–42–45–53–123–281**
B. bifidum	AY004280	22–31–59–181–297
B. bohemicum	**GU223108**	**2–4–14–16–17–31–42–43–45–75–81–200**
B. bombi	EU869281	**27–115–178–281**
B. boum	AY004285	22–117–200–251
B. breve	AF240566	106–139–139–200
B. callithricos	AB674319	**16–22–31–59-462**
B. catenulatum	AY004272	53–198–338
B. choerinum	AY013247	36–42–51–52–54–59–97–200
B. coryneforme	AY004275	16–32–54–158–338
B. cuniculi	AY004283	16–42–53–70–128–281
B. dentium	AF240572	22–31–42–68–130–139–158
B. gallicum	AF240575	42–253–297
B. gallinarum	AY004279	16–31–42–81–139–281
B. indicum	AF240574	16–32–36–42–45–123–296
B. longum subsp. *longum*	AF240578	42–113–138–139–158
B. longum subsp. *infantis*	AF240577	42–113–138–139–158
B. longum subsp. *suis*	AY013248	42–113–138–139–158
B. mongoliense	KF751642[a]	**40–70–78–106–254**
B. merycicum	AY004277	22–31–42–59-139–297
B. minimum	AY004284	16-51–60–66–70–327
B. pseudocatenulatum	AY004274	42–53–198–297
B. pseudolongum subsp pseudolongum	AY004282	17–22–30–32–42–109–297
B. pseudolongum subsp. *globosum*	AF286736	16–17–22–30–32–42–109–323
B. psychraerophilum	AY339132	**No sites**
B. pullorum	AY004278	16–31–36–42–81–87–297
B. reuteri	**AB674318**	**53–59–139–339**
B. ruminantium	AF240571	31–106–114–339
B. saguini	**AB674320**	**53–59–181–297**
B. stellenboschense	**KF294527**[a]	**16–42–53–59–123–139–158**
B. subtile	Not available	Not available
B. thermacidophilum subsp porcinum	AY004276	20–42–53–59–97–139–180
B. thermacidophilum subsp thermacidophilum	AY004276	20–42–53–59–97–139–180
B. thermophilum	AF240567	54–59–117–139–222
B. tsurumiense	**AB241108**	**53–537**
Parascardovia denticolens	**AF240565**	**16–31–36–42–60–81–87-221**
Scardovia inopinata	**AY004281**	**31–559**

Bold font highlights species processed in the present study, and update the list previous published in Baffoni et al. [1].
[a] Obtained by cloning.



a: *B. actinocoliniforme*
b. *B. bohemicum*
c: *B. reuteri*
d: *B. psychraerophylum*
e: *B. bombi*
f: *B. tsurumiense*
g: *B. mongoliense*

h: *B. saguini*
i: *B. stellenboschense*
l: *B. biavatii*
m: *B. callithricos*
n: *Parascardovia denticolens*
o: *Scardovia inopinata*

Fig. 1. Acrylamyde gel electrophoresis of digested *hsp60* DNA fragments with *HaeIII* (negative image).

Table 3
Dichotomous key to identify species of *Bifidobacterium* based upon HaeIII restriction digestion of ~590 bp of the *hsp60* gene.

1. One fragment > 360 bp…… 2
   - No fragment > 360 bp……..4
2. One fragment > 500 bp……3
   - No fragment > 500 bp……*B. callithricos*
3. Fragments at 53 bp……..….*B. tsurumiense*
   - No fragment at 53 bp……..….*Scardovia inopinata*
4. Distinct fragment between 320 and 360 bp……..…..5
   - No fragment between 320 and 360 bp……..12
5. One fragment ≥ 340 bp……..…….....……..7
   - No fragment ≥ 340 bp……..…….....6
6. One fragment at approximately 110 bp ……. *B. pseudolongum* subsp. *globosum*
   - No fragment at approximately 110 bp……..….*B. minimum*
7. Fragments between 280 and 140 bp……..…….8
   - No fragments between 280 and 140 bp……10
8. One fragment at approximately 200 bp……*B. catenulatum*
   - No fragment at approximately 200 bp……9
9. One fragment at approximately 160 bp ……..……..*B. coryneforme*
   - No fragment at approximately 160 bp……..……*B. reuteri*
10. Fragment at approximately 80 bp……*B. adolescentis*
    - No fragment at approximately 80 bp……11
11. One fragment at approximately 30 bp……..……B. *ruminantium*
    - No fragment at approximately 30 bp ..……*B. bombi*
12. Fragment at approximately 280-300 bp……13
    - No fragment at approximately 280-300 bp……..……..25

Table 3 (*continued*)

13.One fragment between 150-260 bp……..16

- No fragment between 150-260 bp……..18

14.Fragment at 250 bp and 240 bp……*B. gallicum*

- No fragment at 250 bp and 240 bp……15

15.Fragment at approximately 240 bp……*B. subtile*

- No fragment at approximately 240 bp……..16

16.Fragment at approximately 200 bp……*B. pseudoctenulatum*

- No fragment at approximately 200 bp……17

17.Fragment at approximately 50 bp……..*B. saguini*

- No fragment at approximately 50 bp……*B. bifidum*

18.Fragment at 140 bp……19

- No fragment at 140 bp……..21

19.One fragment at 80 bp……*B. gallinarum*

- No fragment at 80 bp……20

20.One fragment at 30 bp……*B. merycicum*

- No fragment at 30 bp……..*B. angulatum*

21.Fragment between 100-200 bp……22

- No fragment between 100-200 bp……*B. pullorum*

22.Fragment between 120-130……..23

- No fragment between 120-130 bp…….....……..*B. pseudolongum subsp. pseudolongum*

23.Fragment at approximately 70 bp……..*B. cuniculi*

- No fragment at approximately 70 bp……24

24.Fragment at approximately 30 bp……*B. indicum*

- No fragment at approximately 30 bp……*B. biavatii*

25.Fragment ≥ 220 bp……26

- No fragment ≥ 220 bp……..32

26.At least fragment ≥ 240 bp……..27

- No fragment ≥ 240 bp……..29

27.Fragment at approximately 190 bp……...*B. boum*

- No fragment at approximately 190 bp……...28

*28.*Fragment ≥ 300 bp……*B. asteroides*

- No fragment ≥ 300 bp……..*B. mongoliense*

29.Fragment at approximately 135-140 bp……*B. termophilum*

- No fragment at approximately 135-140 bp……30

30.Fragment at approximately 70 bp……*B. animalis* subsp. *lactis*

- No fragment at approximately 70 bp……31

31.Fragment at 114 bp……*B. animalis* subsp. *animalis*

- No fragment at 114 bp……*Parascardovia denticolens*

32.Fragment at approximately 140 bp……33

- No fragment at approximately 140 bp……..34

33.Fragment at approximately 160 bp……35

- No fragment at approximately 160 bp……...36

34.Fragment near 100 bp……..*B. choerinum*

- No fragment near 100 bp……*B. bohemicum*

(*continued on next page*)

Table 3 (*continued*)

35.Fragment at approximately 70 bp……..*B. dentium*

- No fragment at approximately 70 bp……37

36.Fragment at 40-60 bp……*B. thermoacidophilum* spp.

- No fragment at 40-60 bp……*B. breve*

37.Fragment at approximately 60 bp……*B. stellenboschense*

- No fragment at approximately 60 bp……*B. longum* spp.

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PAPER 2

Modesto M., **Michelini, S.,** Stefanini, I., Ferrara, A., Tacconi, S., Biavati, B. & Mattarelli, P. (2014). *Bifidobacterium aesculapii* sp. nov. from the faeces of the baby common marmoset (*Callithrix jacchus*). *International journal of systematic and evolutionary microbiology*. doi: 10.1099/ijs.0.056937-0.

Bifidobacterium aesculapii sp. nov., from the faeces of the baby common marmoset (*Callithrix jacchus*)

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Six Gram-positive-staining, microaerophilic, non-spore-forming, fructose-6-phosphate phosphoketolase-positive bacterial strains with a peculiar morphology were isolated from faecal samples of baby common marmosets (*Callithrix jacchus*). Cells of these strains showed a morphology not reported previously for a bifidobacterial species, which resembled a coiled snake, always coiled or ring shaped or forming a 'Y' shape. Strains MRM 3/1^T and MRM 4/2 were chosen as representative strains and characterized further. The bacteria utilized a wide range of carbohydrates and produced urease. Glucose was fermented to acetate and lactate. Strain MRM 3/1^T showed a peptidoglycan type unique among members of the genus *Bifidobacterium*. The DNA base composition was 64.7 mol% G+C. Almost-complete 16S rRNA, *hsp60*, *clpC* and *rpoB* gene sequences were obtained and phylogenetic relationships were determined. Comparative analysis of 16S rRNA gene sequences showed that strains MRM 3/1^T and MRM 4/2 had the highest similarities to *Bifidobacterium scardovii* DSM 13734^T (94.6 %) and *Bifidobacterium stellenboschense* DSM 23968^T (94.5 %). Analysis of *hsp60* showed that both strains were closely related to *B. stellenboschense* DSM 23968^T (97.5 % similarity); however, despite this high degree of similarity, our isolates could be distinguished from *B. stellenboschense* DSM 23968^T by low levels of DNA–DNA relatedness (30.4 % with MRM 3/1^T). Strains MRM 3/1^T and MRM 4/2 were located in an actinobacterial cluster and were more closely related to the genus *Bifidobacterium* than to other genera in the family *Bifidobacteriaceae*. On the basis of these results, strains MRM 3/1^T and MRM 4/2 represent a novel species within the genus *Bifidobacterium*, for which the name *Bifidobacterium aesculapii* sp. nov. is proposed; the type strain is MRM 3/1^T (=DSM 26737^T=JCM 18761^T).

Bifidobacteria are Gram-positive, anaerobic, non-motile, non-spore-forming bacteria and represent one of the larger bacterial groups within the *Actinobacteria*. Bifidobacteria are typically found in the gastrointestinal (GI) tracts of humans and other mammals and the hindgut of most social insects, such as honey bees, wasps, cockroaches and bumblebees (Biavati & Mattarelli, 2012; Kopečný *et al.*, 2010; Killer *et al.*, 2009). They are generally host-animal-specific and can be separated into 'human' and 'animal' groups based on their distribution (Ventura *et al.*, 2004). Bifidobacteria are known to exert beneficial effects and to play an important role in maintaining the health of their host (Turroni *et al.*, 2011). Hence, it is important to understand the diversity of bifidobacteria in the GI tract and faeces.

During the characterization of bifidobacterial distribution in primates, six bifidobacterial strains with similar morphology were isolated from fresh faecal samples of baby subjects of the common marmoset (*Callithrix jacchus*), which were individually collected from five animals kept in animal houses at Aptuit s.r.l. Verona, in northern Italy. The common marmoset is a small exudivore monkey from the New World that has developed a large specialized caecum for the digestion of the complex carbohydrates found in tree exudates (Caton *et al.*, 1996; Bailey & Coe, 2002). As microbiota growth and composition are affected by GI tract function, such as motility and nutrient availability in the intestinal lumen, it is likely that this evolutionary adaptation may influence the concentrations and types of

Abbreviation: F6PPK, fructose-6-phosphate phosphoketolase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial *hsp60*, *rpoB* and *clpC* gene sequences of strain MRM 3/1^T are KC807989, KC997237, KC997239 and KF164211 and those of strain MRM4/2 are KC807990, KC997238, KC997240 and KF164212, respectively. The accession number for the partial *hsp60* gene sequence of *B. scardovii* DSM 13734^T is KJ689460.

Four supplementary figures are available with the online version of this paper.

bacteria that form part of the normal intestinal microbiota (Bailey & Coe, 2002).

Samples of fresh rectal swabs from common marmosets were serially diluted with peptone water (Merck) supplemented with cysteine hydrochloride (0.5 g l^{-1}); aliquots of each dilution were inoculated onto TPY agar supplemented with mupirocine (100 mg l^{-1}; Applichem), which is a selective agent for bifidobacteria (Rada & Petr, 2000). In each subject, we observed cells of a bacterium with a novel and unusual morphology, resembling a coiled snake. A total of six isolates with this morphology were obtained from the five baby marmosets. They were namely MRM 3/1^T, MRM 4/2, MRM 5/13, MRM 8/7, MRM 4/6 and MRM 4/7. The isolates were subcultured on TPY agar and cells were suspended in a 10 % (w/v) sterile skimmed milk solution supplemented with lactose (3 %) and yeast extract (0.3 %) and kept both freeze-dried and frozen at −120 °C. For all experiments, the strains were cultivated under anaerobic conditions and maintained in TPY broth, pH 6.9, at 37 °C, unless indicated otherwise.

In the present study, the morphological, biochemical and molecular characterization of the isolates was carried out.

Chromosomal DNA was obtained from the isolates according to the procedure of Rossi *et al.* (2000), with slight modifications. Briefly, cells of overnight cultures were pelleted and resuspended in 1 ml TE buffer (pH 7.6) containing 50 mg lysozyme ml^{-1} and then incubated overnight at 37 °C.

For discrimination of the isolates, molecular typing was performed using enterobacterial repetitive intergenic consensus sequences (ERIC) PCR with the primers ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Ventura *et al.*, 2003). Each 20 μl reaction mixture contained 3.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 μM each dNTP (HotStarTaq plus DNA polymerase MasterMix kit; Qiagen), 30 ng DNA template and 2 μM each primer. Amplifications were performed using an Applied Biosystems Veriti Thermal Cycler with the following temperature profile: 1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 4 min; and 1 cycle at 72 °C for 6 min. Aliquots of each amplification reaction mixture (15 μl each) were separated by electrophoresis in 2 % (w/v) agarose gels at a voltage of 7 V cm^{-1}. Gels were stained with ethidium bromide (0.5 μg ml^{-1}) and photographed under 260 nm UV light. Given that the isolates revealed two different ERIC-PCR profiles (Fig. S1, available in the online Supplementary Material), strains MRM 3/1^T and MRM 4/2 were selected as representatives and further characterized.

The partial 16S rRNA genes of strains MRM 3/1^T and MRM 4/2 were amplified by PCR using the primers Bif285 (5′-GAGGGTTCGATTCTGGCTCAG-3′) and Bif261 (5′-AAGGAGGTGATCCAGCCGCA-3′) (Kim *et al.*, 2010). Partial *hsp60*, *rpoB* and *clpC* gene sequences were also obtained using the primer pairs HspF3 (5′-ATCGCCAAGGAGATCGAGCT-3′) and HspR4 (5′-AAGGTGCCGCGGATCTTGTT-3′), BifF (5′-TCGATCGGGCACATACGG-3′) and BifR2 (5′-CGACCACTTCGGCAACCG-3′) (Kim *et al.*, 2010) and BClpC-F (5′–ATCGCSGARACBATYGAGA-3′) and BClpC-R (5′-ATRATGCGCTTGTGCARYT-3′) (Watanabe *et al.*, 2009), respectively. Each PCR mixture (20 μl) contained 1.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 μM each dNTP (HotStarTaq plus DNA polymerase MasterMix kit; Qiagen), 0.1 μM each primer and 30 or 200 ng DNA template for the 16S rRNA gene and for each housekeeping gene, respectively. Amplifications were performed using a TGradient thermal cycler (Biometra). A touchdown PCR was used to amplify the 16S rRNA gene and the other phylogenetic markers as follows: initial denaturation (95 °C, 5 min) for HotStarTaq plus activation; four cycles of denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s and extension at 72 °C for 90 s; 21 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 90 s; and 15 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. The PCR was completed with a single elongation step (10 min at 72 °C). The resulting amplicons were separated on 2 % agarose gels, followed by ethidium bromide staining. PCR fragments were purified using the NucleoSpin extract II kit (Macherey-Nagel) following the manufacturer's instructions.

16S rRNA genes were directly sequenced whereas *hsp60*, *clpC* and *rpoB* gene sequences were cloned using an InsTAclone PCR Cloning kit (Fermentas). All sequencing reactions were performed by Eurofins MWG Operon. Almost-complete 16S rRNA gene sequence assembly was performed using CAP (contig assembly program; Huang, 1992) in BioEdit (Hall, 1999). After editing, the closest known relatives of the novel strains were determined by comparison with database entries and the sequences of closely related strains were retrieved from the EMBL and GenBank nucleotide databases. Pairwise nucleotide sequence similarity values were calculated using the EzTaxon server (http://www.eztaxon.org/), which provides a web-based tool (Kim *et al.*, 2012).

The 16S rRNA gene sequences (about 1421 bp) of strains MRM 3/1^T and MRM 4/2 and of those of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned by using the CLUSTAL_X2 program (version 1.82) (Thompson *et al.*, 1997). A phylogenetic tree based on a total of 43 partial 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium* and of related genera, was reconstructed with the neighbour-joining method (Saitou & Nei, 1987) and evolutionary distances were computed using Kimura's two-parameter method (Kimura, 1980) by using the MEGA 5.05 program (Tamura *et al.*, 2011). The tree was rooted using *Micrococcus luteus* DSM 20030^T (Fig. 1). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985) and the tree topology was also confirmed with the maximum-likelihood (Cavalli-Sforza & Edwards, 1967), maximum-parsimony (Fitch, 1971) and least-squares (Fitch & Margoliash, 1967) methods, by using MEGA 5.05

(Tamura *et al.*, 2011). The 16S rRNA gene sequence similarity between strains MRM 3/1^T and MRM 4/2 was about 99.6 %. They showed low sequence similarity to known bifidobacteria; the highest similarities were found to the type strains of *Bifidobacterium scardovii* and *Bifidobacterium stellenboschense* (94.6 and 94.5 %, respectively), a recently described species from a red-handed tamarin (*Saguinus midas*) (Endo *et al.*, 2012). Based on the neighbour-joining analysis, the novel strains are related phylogenetically to *B. scardovii* (Fig. 1). Similar tree topologies were obtained by using the maximum-likelihood (Fig. S2), maximum-parsimony and least-squares methods (not shown).

Multilocus sequence analysis is a reliable and robust technique for the identification and classification of bacterial isolates to the species level as an alternative to 16S rRNA gene sequence analysis (Martens *et al.*, 2008). For this reason, the phylogenetic location of the novel strains was verified by analysis of three additional phylogenetic markers, *hsp60*, *clpC* and *rpoB*, which have proven to be discriminative for classification of the genus *Bifidobacterium* (Jian *et al.*, 2001; Ventura *et al.*, 2006; Kim *et al.*, 2010).

For *hsp60*, *clpC* and *rpoB* genes, the sequences of strains MRM 3/1^T and MRM 4/2 and of those of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned by using the MAFFT program, at CBRC (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). The Gblocks program (version 0.91b) as server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/Gblocks.html) was then used to eliminate poorly aligned positions and divergent regions of DNA alignments, so that they became more suitable for phylogenetic analysis (Talavera & Castresana, 2007).

To complete our phylogenetic determination, the partial *hsp60* gene was amplified, purified and directly sequenced from *B. scardovii* DSM 13734^T as described above, whereas, for *B. stellenboschense* DSM 23968^T, we used the partial gene sequence obtained by Stenico *et al.* (2014) and retrieved from GenBank.

Three phylogenetic trees were then reconstructed using the neighbour-joining method. Approximately 645 bp of the *hsp60* gene, 500 bp of the *clpC* gene and 524 bp of the *rpoB* gene sequence of the isolates and related strains were used in the analyses.

The level of similarity for the partial *hsp60* gene sequences of strains MRM 3/1^T and MRM 4/2 was 99.5 % and, in relation to the type strains of their closest relatives, the levels of similarity were about 97.5 % with *B. stellenboschense*, 96.2 % with *Bifidobacterium saeculare*, 96 % with *Bifidobacterium pullorum* and *Bifidobacterium gallinarum*, 94.4 % with *Bifidobacterium biavatii*, 94 % with *Bifidobacterium callitrichos* and 90.8 % with *B. scardovii*. Strains MRM 3/1^T and MRM 4/2 formed a subcluster in the *B. pullorum* group (Fig. 2).

The sequence similarity between the *clpC* genes of strains MRM 3/1^T and MRM 4/2 was 99.2 %. The highest sequence similarities were found to the type strains of *B. scardovii* and *Bifidobacterium bifidum* (about 86.7 and 86.5 %, respectively). Strains MRM 3/1^T and MRM 4/2 produced a subcluster in the *B. scardovii* group.

The *clpC* phylogenetic tree is shown in Fig. S3.

The level of similarity for the partial *rpoB* gene sequences of strains MRM 3/1^T and MRM 4/2 was 99.8 %, and the levels of similarity in relation to their closest relatives were about 95.2, 95 and 94 % to the type strains of *Bifidobacterium cuniculi*, *Bifidobacterium choerinum* and *B. pullorum*, respectively. Based on the partial *rpoB* sequences, MRM 3/1^T and MRM 4/2 are placed in a distinct cluster and were related to *B. cuniculi*. The *rpoB* phylogenetic tree is shown in Fig. S4.

These findings correlated with the results of Ventura *et al.* (2006) and Endo *et al.* (2012) and indicated that the phylogenetic positions of species of the genus *Bifidobacterium* are highly influenced by the genes used for the analysis.

The 16S rRNA gene sequence similarity of strains MRM 3/1^T and MRM 4/2 to known species was less than 97 % and it was lower than the recommended value for species differentiation (98.7–99 %; Tindall *et al.*, 2010). However, analysis of *hsp60* showed that both strains were closely related to *B. stellenboschense* DSM 23968^T (97.5 % similarity). Due to this high level of similarity (the cut-off value for bifidobacterial species differentiation of *hsp60* is 96 %; Zhu *et al.*, 2003), DNA–DNA hybridization between strain MRM 3/1^T and *B. stellenboschense* DSM 23968^T was also performed. Estimation of the level of relatedness between *B. stellenboschense* DSM 23968^T and strain MRM 3/1^T was determined by the DSMZ, Braunschweig, Germany. Cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). Strain MRM 3/1^T shared 30.4 % DNA–DNA relatedness with *B. stellenboschense* DSM 23968^T, unequivocally supporting the assignment of strain MRM 3/1^T to a novel species.

Estimation of the G+C content in bacterial chromosomal DNA of strain MRM 3/1^T was done by the DSMZ. DNA was purified on hydroxyapatite according to the procedure of Cashion *et al.* (1977) and enzymically hydrolysed by the method of Mesbah *et al.* (1989). The resulting deoxyribonucleosides were analysed by HPLC as described by Tamaoka & Komagata (1984). Strain MRM 3/1^T had a DNA G+C content of 64.7 mol%. This value was within the range of DNA G+C content reported for the genus *Bifidobacterium*, 52–67 mol% (Biavati & Mattarelli, 2012; Killer *et al.*, 2010), and in particular was very similar to that

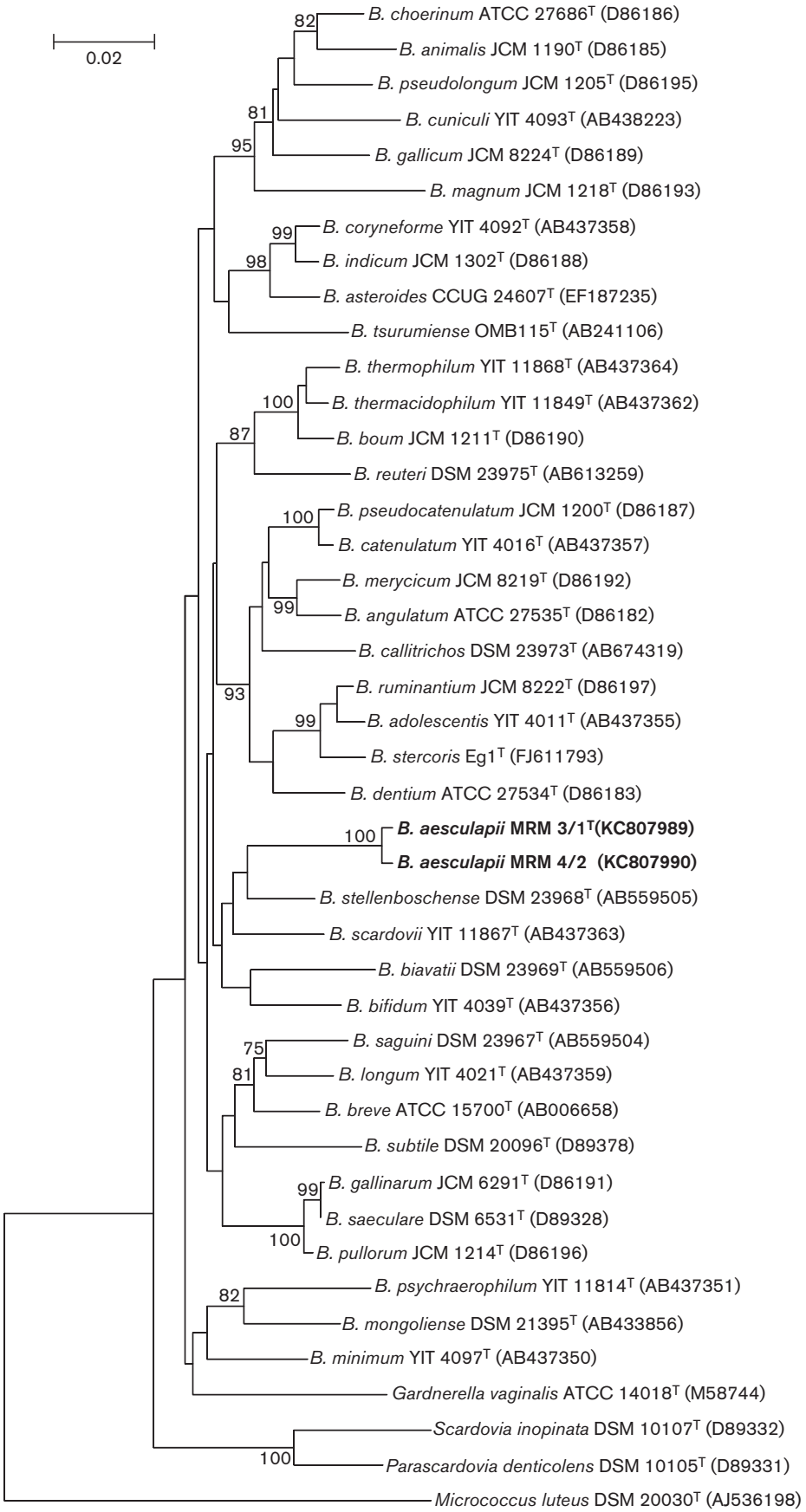

Fig. 1. Phylogenetic relationships of the novel bifidobacteria to related species based on 16S rRNA gene sequences. The tree was reconstructed by the neighbour-joining method and rooted with *Micrococcus luteus* DSM 20030^T. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. Bootstrap values above 70 % are given at branching points. Bar, 0.02 substitutions per nucleotide position.

obtained for *B. callitrichos*, described recently from a marmoset by Endo *et al.* (2012).

Morphological, cultural and biochemical characterization of the isolates according to standard techniques was performed at 37 °C unless otherwise stated. Morphology as examined by phase-contrast microscopy is shown in Fig. 3(a, b). Morphological characteristics determined using a scanning electron microscope (SEM) are shown in Fig.

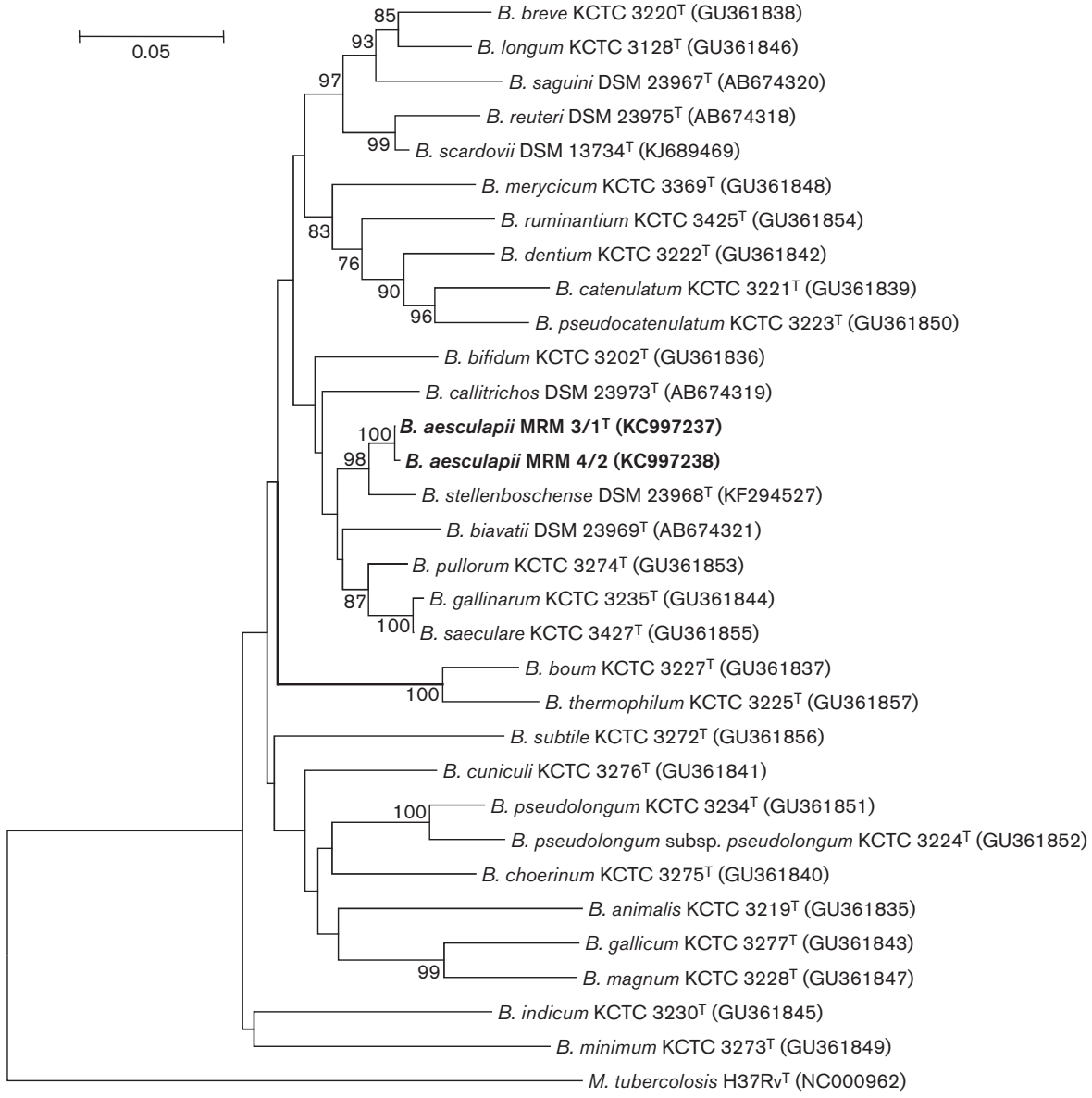

Fig. 2. Phylogenetic tree based on *hsp60* gene sequences showing the relationships of the novel strains isolated from baby marmosets to closely related species. The tree was reconstructed by the neighbour-joining method on the basis of a comparison of 559 positions, and the sequence of *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. Bootstrap values above 70 % are given at branching points. Bar, 0.05 substitutions per nucleotide position.

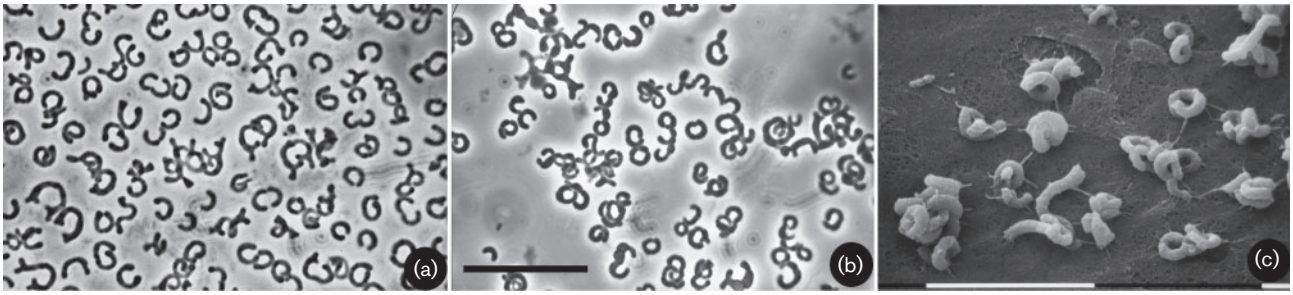

Fig. 3. Cellular morphology of cells grown in TPY broth. (a, b) Phase-contrast photomicrographs of strains MRM 3/1^T (a) and MRM 4/2 (b). Bar, 10 μm (b). (c) Scanning electron photomicrograph of a cell of strain MRM 3/1^T. Bar, 10 μm.

3(c). For SEM observation, strains were cultured on TPY agar at 37 °C for 48 h under anaerobic conditions. After culturing, a slice of agar was excised and dehydrated with a series of increasing ethanol concentrations (50, 70, 80, 90, 95 and 100 % for 15 min each). The prepared cells were subsequently critical-point-dried in a critical point dryer apparatus (CPD Emitech K850) using liquid CO_2 as a transitional fluid. Dried samples were mounted on aluminium stubs with silver glue, coated with gold palladium film using an ion-sputtering unit (Emitech K500) and observed in a Philips 515 SEM at 7–10.0 kV.

The temperature range for growth of the strains was tested using an anaerobic TPY broth at 20, 25, 30, 35, 37, 40, 42, 45 and 47 °C for 48 h. The sensitivity of the strains to low pH was determined at 37 °C in anaerobic TPY broth (pH 3.5, 4.0, 4.5, 5.0 and 5.5) for 48 h. The ability of the strains to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was tested using TPY agar, TPY soft agar (0.6 %), TPY broth, skimmed milk and UHT whole milk at 37 °C for 48 h.

Haemolytic activity was determined in Columbia blood agar (Biolife) at 37 °C under anaerobic conditions for 48 h (Pineiro & Stanton, 2007).

Spore staining was performed using malachite green dye. Phase-contrast microscopy (Zeiss) was used to observe the morphology of individual cells as well as spore staining.

Gram staining and catalase and oxidase activities were respectively determined from cells grown on TPY agar at 37 °C for 48 h under anaerobic conditions using Gram staining individual reagents (Merck Millipore), a 3 % (v/v) hydrogen peroxide solution and cotton swabs impregnated with *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine dihydrochloride and dried (Oxibioswab; Biolife). The motility of strains was determined by stabbing into TPY medium containing 0.4 % agar, knowing that motile strains show diffuse growth spreading from the line of inoculation. Fermentation products (short-chain fatty acids) were analysed according to the method described by Holdeman *et al.* (1977). Briefly, after growth in TPY broth with 1 % glucose, volatile acids were extracted with diethyl ether. A Carlo Erba 5300 gas chromatograph, with a Nukol capillary column (30 cm) at 170 °C, flame-ionization detector and hydrogen carrier gas, was used for the analysis. All strains tested fermented glucose to acetate and lactate in a variable ratio ranging from 2 : 1 to 1.5 : 1.

Biochemical characterization was carried out by using the API 20A, API 20E and API 50CHL systems (bioMérieux) following the manufacturer's instructions. The results are summarized in Table 1.

Bifidobacteria and members of related genera degrade hexoses via the fructose-6-phosphate phosphoketolase (F6PPK) pathway. F6PPK is the key enzyme in this pathway and is considered a taxonomic marker for identification of species of *Bifidobacterium* and related genera (Biavati & Mattarelli, 2012). F6PPK activity was determined according to the method described by Scardovi (1986) and modified by Orban & Patterson (2000). All the isolates possessed F6PPK activity.

The cell-wall murein composition of strain MRM 3/1^T was examined by the DSMZ. Analysis of partial acid hydrolysates revealed the presence of A4α-type, L-Lys–D-Ser–D-Asp. This murein type is unique among members of the genus *Bifidobacterium* and related genera, confirming the novelty of this species.

According to our phylogenetic analyses based on 16S rRNA gene and partial *hsp60*, *clpC* and *rpoB* sequences and the other data obtained, strains MRM 3/1^T, MRM 4/2, MRM 5/13, MRM 4/6, MRM 4/7 and MRM 8/7 are genetically and phenotypically distinguishable from currently recognized species of bifidobacteria and thus represent a novel species, for which we suggest the name *Bifidobacterium aesculapii* sp. nov.

Description of *Bifidobacterium aesculapii* sp. nov.

Bifidobacterium aesculapii (aes.cu.la′pi.i. L. gen. masc. n. *aesculapii* of Aesculapius, from the snake-like appearance of the bacterium, resembling the serpent-entwined rod wielded by the Roman god Aesculapius).

Cells grown in TPY broth are rods of various shapes, occasionally swollen, always coiled or ring shaped or

Table 1. Differential characteristics between the novel bifidobacteria and their closest phylogenetic relatives

Strains: 1, MRM 3/1^T; 2, MRM 4/2; 3, MRM 8/7; 4, MRM 5/13; 5, MRM 4/6; 6, MRM 4/7; 7, *B. stellenboschense* DSM 23968^T; 8, *B. biavatii* DSM 23967^T; 9, *B. scardovii* DSM 13734^T; 10, *B. bifidum* DSM 20456^T. All data were obtained in this study unless indicated. +, Positive, w, weakly positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10
Utilization of:										
D-Mannitol	+	−	−	w	−	+	w	w	−	−
Sucrose	+	−	+	+	−	+	+	+	+	+
Maltose	+	+	+	w	+	+	+	+	+	−
Salicin	+	+	+	−	+	+	+	+	−	−
D-Xylose	+	+	+	+	+	+	+	+	−	−
L-Arabinose	+	+	+	+	+	+	w	+	−	−
Glycerol	+	−	−	−	−	+	+	w	−	+
Cellobiose	+	−	−	−	−	+	−	+	−	−
D-Mannose	+	−	−	−	−	+	−	w	−	−
Melezitose	+	w	w	w	w	+	w	+	−	−
Raffinose	+	w	+	+	−	+	+	+	+	−
D-Sorbitol	+	w	+	+	+	+	w	w	−	−
L-Rhamnose	+	−	−	−	−	+	−	w	−	−
Trehalose	+	−	−	−	−	+	−	+	−	−
D-Ribose	w	+	+	w	+	+	+	+	−	−
D-Galactose	w	+	+	+	+	+	+	+	−	+
D-Fructose	−	+	+	+	+	+	+	+	+	−
Starch	+	−	−	−	w	−	−	−	+	−
Gentiobiose	+	w	+	+	+	+	w	+	+	−
D-Turanose	w	+	+	+	+	+	+	+	w	−
Arbutin	+	+	+	+	+	+	+	+	−	−
Melibiose	w	w	+	+	+	w	+	+	w	−
Inulin	w	−	w	w	w	w	−	−	−	−
Potassium gluconate	w	w	+	−	w	+	w	w	−	−
Glycogen	+	−	−	−	−	−	−	w	+	−
Xylitol	w	−	−	−	−	−	−	+	−	−
Amygdalin	−	−	−	−	−	−	+	+	−	−
Methyl α-D-glucopyranoside	−	−	−	−	−	−	+	−	−	−
N-Acetylglucosamine	−	−	−	−	−	−	+	−	−	−
DNA G+C content (mol%)	64.7	ND	ND	ND	ND	ND	66.3*	60.1*	63.1*	58*
Urease activity	+	+	+	+	+	+	−	−	−	−

*Data from Endo *et al.*, 2012.

forming a 'Y' shape at both ends. They are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and microaerophilic. There is no difference in growth under either anaerobic or microaerophilic conditions. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization and H_2S production. Does not reduce nitrate or nitrite. Well-isolated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while imbedded colonies are lens-shaped or elliptical. Colonies reach 1.7–2.5 mm in diameter after 3 days of incubation. The temperature range for growth is 25–42 °C; no growth occurs at 20 or 47 °C. The optimum temperature for growth is 35–37 °C. Grows at pH 4.5–7.0 with an optimum at pH 6.5–7.0. Can grow in milk, under aerobic, microaerophilic and anaerobic conditions. Acid is produced from D-glucose, lactose, maltose, salicin, D-xylose, L-arabinose, melezitose, D-sorbitol, D-ribose, D-galactose, gentiobiose, D-turanose, arbutin, melibiose and potassium gluconate. Acid production from D-mannitol, sucrose, glycerol, cellobiose, D-mannose, raffinose, L-rhamnose, trehalose, D-fructose, starch, inulin and glycogen is strain dependent. Acid is not produced from xylitol, amygdalin, methyl α-D-glucopyranoside, *N*-acetylglucosamine or potassium gluconate. Lactic and acetic acids are produced as end products of glucose fermentation in a variable ratio ranging from 1 : 2 to 1 : 1.5. Aesculin is hydrolysed and urease is produced. The peptidoglycan type is A4α L-Lys–D-Ser–D-Asp. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. scardovii* subgroup of the genus *Bifidobacterium*.

The type strain, MRM 3/1^T (=JCM 18761^T=DSM 26737^T), and the reference strain MRM 4/2 (=JCM 18762=DSM 26738) were isolated from fresh faecal samples of infant common marmosets (*Callithrix jacchus*) that were individually collected from animals kept in animal houses in Aptuit s.r.l. Verona, northern Italy, in 2012. The DNA G+C content of the type strain is 64.7 mol%.

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PAPER 3

Modesto, M., **Michelini, S.,** Stefanini, I., Sandri, C., Spiezio, C., Pisi, A., Filippini, G., Biavati, B. & Mattarelli, P. (2015). *Bifidobacterium lemurum* sp. nov. from the faeces of the ring-tailed lemur (*Lemur catta*). *International journal of systematic and evolutionary microbiology*. doi: 10.1099/ijs.0.000162.

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Bifidobacterium lemurum sp. nov., from faeces of the ring-tailed lemur (*Lemur catta*)

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Four Gram-positive-staining, microaerophilic, non-spore-forming, fructose-6-phosphate phosphoketolase-positive bacterial strains were isolated from a faecal sample of a 5-year-old ring-tailed lemur (*Lemur catta*). The strains showed a peculiar morphology, resembling a small coiled snake, a ring shape, or forming a little 'Y' shape. The isolated strains appeared identical, and LMC 13^T was chosen as a representative strain and characterized further. Strain LMC 13^T showed an A3β peptidoglycan type, similar to that found in *Bifidobacterium longum*. The DNA base composition was 57.2 mol% G+C. Almost-complete 16S rRNA, *hsp60*, *rpoB*, *dnaJ*, *dnaG*, *purF*, *clpC* and *rpoC* gene sequences were obtained, and phylogenetic relationships were determined. Comparative analysis of 16S rRNA gene sequences showed that strain LMC 13^T showed the highest similarity to *B. longum* subsp. *suis* ATCC 27533^T (96.65 %) and *Bifidobacterium saguini* DSM 23967^T (96.64 %). Strain LMC 13^T was located in an actinobacterial cluster and was more closely related to the genus *Bifidobacterium* than to other genera in the *Bifidobacteriaceae*. On the basis of these results, strain LMC 13^T represents a novel species within the genus *Bifidobacterium*, for which the name *Bifidobacterium lemurum* sp. nov. is proposed; the type strain is LMC 13^T (=DSM 28807^T=JCM 30168^T).

Bifidobacteria are Gram-positive, anaerobic, non-motile and non-spore-forming bacteria and represent one of the large bacterial groups within the class *Actinobacteria*. Members of the genus *Bifidobacterium* are typically found in the gastrointestinal tracts (GIT) of humans and other mammals and the hindguts of honeybees and bumblebees (Biavati & Mattarelli, 2012; Killer *et al.*, 2009, 2011; Turroni *et al.*, 2014; Ventura *et al.*, 2007, 2012). They have also been isolated from waste and dairy products, where the source could have been faecal contamination and intentional probiotic addition, respectively (Mattarelli & Biavati, 2014).

The occurrence and species composition of bifidobacteria in different animals are quite variable; indeed, they are generally host-animal-specific micro-organisms that can be separated into 'human' and 'animal' groups (Ventura *et al.*, 2004). Bifidobacteria are well known for their beneficial effects, and play an important role in maintaining the health of their hosts (Turroni *et al.*, 2011). Thus, the discovery of bifidobacterial diversity in the GIT, together with the isolation and characterization of novel bacterial taxa in different hosts, is important from the viewpoint of their potential to benefit the health of both humans and economically important animals (Killer *et al.*, 2014).

Yildirim *et al.* (2010) characterized the faecal microbiome from non-human wild primates, and found compelling evidence that, apart from diet, differences in the microbiome species of the different primate families could not be accounted for solely by habitat conditions; in fact, it has been revealed that, in the course of evolution, primate gastrointestinal microbiomes became linked, functionally, to their vertebrate host taxa and are, perhaps, host-specific (Yildirim *et al.*, 2010).

During the study of bifidobacterial distribution in non-human primates, four bifidobacterial strains with the same morphology were isolated from fresh faecal samples of an adult ring-tailed lemur (*Lemur catta*) housed under

Abbreviations: F6PPK, fructose-6-phosphate phosphoketolase; GIT, gastrointestinal tract.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and partial *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *purF*, *clpC* and *rpoC* gene sequences of strain LMC 13^T are KJ658281–KJ658286, KJ960215 and KJ960216, respectively. Accession numbers for the partial *dnaG*, *purF*, *rpoC* and *dnaJ* gene sequences of *B. aesculapii* DSM 26737^T are respectively KP284857–KP284860.

Ten supplementary figures are available with the online Supplementary Material.

semi-natural conditions at Parco Natura Viva, Verona, northern Italy.

Ring-tailed lemurs are strepsirrhine primates endemic to Madagascar. They are described as generalist feeders, and have a pronounced seasonal foraging strategy that results in periodic dietary changes (Campbell *et al.*, 2000). Such lemurs can best be characterized as opportunistic omnivores with a wide dietary regime, which includes fruit, leaves, leaf stems, flowers, flower stems, spiders, spider webs, caterpillars, cicadas, insect cocoons and sometimes birds (Gould, 2006; Jolly *et al.*, 2006).

Dietary specialization in lemur species is always correlated with significant differences in GIT morphology. Indeed, *Lemur catta* shows a somewhat enlarged haustrated caecum, a common adaptation to an herbivorous diet. This caecum harbours an intestinal symbiotic microbiota, and it has been assumed that this facilitates plant cell-wall breakdown and leaf fermentation (Campbell *et al.*, 2000; Jolly *et al.*, 2006).

In February 2014, fresh ring-tailed lemur faeces were collected from the ground using a sterile spoon, put into a sterile plastic tube and stored under anaerobic conditions in an anaerobic jar (Merck) at 4 °C. Samples of fresh faeces were collected by the animal-care staff (keepers) during their routine cleaning of the enclosure, and were taken promptly to the laboratory (within 2 h) Samples of the material, of approx. 1–2 g, were serially diluted with peptone water (Merck) supplemented with cysteine hydrochloride (0.5 g l^{-1}), and aliquots of each dilution were inoculated onto TOS agar (Sigma Aldrich). We observed cells of a bacterium with a morphology resembling a small coiled snake or a little ring, very similar to, but smaller than, that of *Bifidobacterium aesculapii*, a species we recently described in baby common marmoset (Modesto *et al.*, 2014).

A total of four isolates with this morphology were obtained from this one adult ring-tailed lemur subject, and were named LMC 13^T, LMC 16, LMC 18 and LMC 19. They were then subcultured on TPY and cells were suspended in a 10 % (w/v) sterile skimmed milk solution supplemented with lactose (3 %) and yeast extract (0.3 %), freeze-dried and kept frozen at −120 °C. For all experiments, the strains were cultivated under anaerobic conditions in anaerobic jars (Merck) and maintained in TPY broth, at pH 6.9 and 37 °C, unless indicated otherwise. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD).

Chromosomal DNA was obtained from the isolates according to the procedure of Rossi *et al.* (2000), with slight modifications. Briefly, cells of overnight cultures were pelleted and resuspended in 1 ml TE buffer (pH 7.6) containing 50 mg lysozyme ml^{-1} and then incubated overnight at 37 °C.

For isolate discrimination, molecular typing was performed using enterobacterial repetitive intergenic consensus sequence (ERIC) PCR with the primer pair ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Ventura *et al.*, 2003). Each 20 µl reaction mixture contained 3.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStart *Taq* plus DNA polymerase MasterMix kit; Qiagen), 30 ng DNA template and 2 µM each primer. Amplifications were performed using an Applied Biosystems Veriti thermal cycler with the following temperature profile: 1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 4 min; and 1 cycle at 72 °C for 6 min. Aliquots (15 µl each) of each amplification reaction mixture were separated by electrophoresis in 2 % (w/v) agarose gels at a voltage of 7 V cm^{-1}. Gels were stained with ethidium bromide (0.5 µg ml^{-1}) and photographed under 260 nm UV light.

Given that the isolates revealed identical ERIC profiles (see Fig. S1, available in the online Supplementary Material), strain LMC 13^T was selected as a representative and characterized further. Morphological, biochemical and molecular characterizations were carried out on this isolate.

The partial 16S rRNA gene of strain LMC 13^T was amplified by PCR using the primer pair Bif285 (5′-GAGGGTTCGATTCTGGCTCAG-3′) and Bif261 (5′-AAGGAGGTGATCCAGCCGCA-3′) (Kim *et al.*, 2010). Partial *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *purF*, *clpC* and *rpoC* gene sequences were also obtained using the primer pairs HspF3 (5′-ATCGCCAAGGAGATCGAGCT-3′) and HspR4 (5′-AAGGTGCCGCGGATCTTGTT-3′), BifF (5′-TCGATCGGGCACATACGG-3′) and BifR2 (5′-CGACCACTTCGGCAACCG-3′) (Kim *et al.*, 2010), DnaG-uni (5′-CTGTGCCCGTTCCACGAC-3′) and DnaG-rev (5′-CTCGATGCGCAGGTCGCA-3′), DnaJ1-uni (5′-GAGAAGTTCAAGGACATCTC-3′) and DnaJ1-rev (5′-GCTTGCCCTTGCCGG-3′), PurF-uni (5′-CATTCGAACTCCGACACCGA-3′) and PurF-rev (5′-GTGGGGTAGTCGCCGTTG-3′), ClpC-uni (5′-GAGTACCGCAAGTACATCGAG-3′) and ClpC-rev (5′-CATCCTCATCGTCGAACAGGAAC-3′), and RpoC-uni (5′-GTGCACTCGGTCCACAG-3′) and RpoC-rev (5′-CATGCTCAACAACGAGAAG-3′) (Ventura *et al.*, 2006), respectively.

Each PCR mixture (20 µl) contained 1.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStartTaq plus DNA polymerase MasterMix kit; Qiagen), 0.1 µM each primer and 30 or 200 ng DNA template for the 16S rRNA gene and for each housekeeping gene, respectively. Amplifications were performed using a TGradient thermal cycler (Biometra). A touchdown PCR was used to amplify the 16S rRNA gene and all phylogenetic markers (*hsp60*, *rpoB*, *rpoC*, *dnaJ*, *dnaG*, *clpC* and *purF*), and was performed as follows: initial denaturation (95 °C, 5 min) for HotStartTaq plus activation; 4 cycles of denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s and extension at 72 °C for 90 s; 21 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 90 s; and 15 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. The PCR was completed with a single elongation step (10 min at 72 °C).

All the resulting amplicons were separated on 2 % agarose gels, followed by ethidium bromide staining. PCR fragments were purified using the NucleoSpin gel and PCR clean up kit (Macherey-Nagel) following the manufacturer's instructions.

To infer a correct phylogeny, the 16S rRNA gene was cloned using an InsTAclone PCR Cloning kit (Fermentas), whereas the partial *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *purF*, *clpC* and *rpoC* genes were sequenced directly. All sequencing reactions were performed by Eurofins MWG Operon. Assembly of the almost-complete 16S rRNA gene sequence was performed with the BioEdit program (Hall, 1999).

After editing, the closest known relatives of the novel strains were determined by comparison with database entries, and sequences of members of closely related species were retrieved from the EMBL and GenBank nucleotide databases. Pairwise nucleotide sequence similarity values were calculated using the LALIGN program (http://embnet.vital-it.ch/software/LALIGN_form.html), which provides a web-based tool.

The sequencing of 15 clones containing 16S rRNA genes did not reveal any heterogeneity of the rRNA operons within the genome of strain LMC 13^T. The 16S rRNA gene sequences (about 1400 bp) of strain LMC 13^T and its closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned using the CLUSTAL OMEGA program as a web service from the EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/) (McWilliam *et al.*, 2013). A phylogenetic tree based on a total of 48 available partial 16S rRNA gene sequences of members of the genus *Bifidobacterium* was reconstructed with the neighbour-joining method (Saitou & Nei, 1987), and evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980) using MEGA version 6.0 (Tamura *et al.*, 2013). The tree was rooted using *Mycobacterium tuberculosis* H37Rv^T (Fig. 1). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985), and tree topology was confirmed with the maximum-likelihood method (Cavalli-Sforza & Edwards, 1967) using MEGA version 6.0 (Tamura *et al.*, 2013). Strain LMC 13^T showed low sequence similarities to known bifidobacteria (Table 1), and the highest values were found to *Bifidobacterium longum* subsp. *suis* ATCC 27533^T and *Bifidobacterium saguini* DSM 23967^T (96.65 and 96.64 %, respectively); the latter strain belongs to a species described recently from red-handed tamarin (*Saguinus midas*) by Endo *et al.* (2012). Based on the neighbour-joining analysis, the novel strain is related phylogenetically to *B. longum* subsp. *suis* (Fig. 1). Similar tree topologies were obtained by the maximum-likelihood method (Fig. S2).

Multilocus sequence analysis is a reliable and robust technique for the identification and classification of bacterial isolates to the species level, as an alternative or complement to 16S rRNA gene sequence analysis (Martens *et al.*, 2008). Thus, the phylogenetic location of the novel strain was verified by the analysis of seven additional genetic markers, *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *purF*, *clpC* and *rpoC*, which have proven to be discriminative for the classification of the genus *Bifidobacterium* (Jian *et al.*, 2001; Kim *et al.*, 2010; Ventura *et al.*, 2006).

The sequences of the *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *purF*, *clpC* and *rpoC* genes of strain LMC 13^T were amplified and sequenced. Sequences from the type strains of 48 bifidobacterial taxa were retrieved from the public database of the National Center for Biotechnology Information (NCBI). The *purF* analysis included only 47 type strains as, despite several unsuccessful efforts using different PCR amplification parameters, we could not obtain a specific amplicon from *Bifidobacterium actinocoloniiforme* DSM 22766^T, and it was missing from the whole-genome shotgun project (GenBank accession no. JGYK00000000). To complete the phylogenetic study, partial *dnaG*, *purF*, *rpoC* and *dnaJ* gene sequences were amplified and sequenced directly from *Bifidobacterium aesculapii* DSM 26737^T.

Sequences were aligned using the MAFFT program at CBRC (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). The Gblocks program (version 0.91b), a server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was then used to eliminate poorly aligned positions and divergent regions of DNA alignments, facilitating the phylogenetic analysis (Talavera & Castresana, 2007).

Approximately 594 bp of the *hsp60* gene, 526 bp of the *rpoB* gene, 933 bp of the *dnaG* gene, 488 bp of the *dnaJ* gene, 930 bp of the *purF* gene, 1171 bp of the *rpoC* gene and 717 bp of the *clpC* gene sequences of strain LMC 13^T and type strains of related species were used in the analyses. Seven phylogenetic trees were then produced using the individual genes (Figs 2 and S3–S8). The levels of similarity for the eight partial gene sequences obtained from strain LMC 13^T and its closest relatives are summarized in Table 1.

The concatenation of gene sequences has been shown to be extremely useful in order to infer bacterial phylogeny (Ventura *et al.*, 2006). For this purpose, an additional tree, including 47 bifidobacterial type strains, was created on the basis of the concatenation of all housekeeping gene sequences that we were able to retrieve or sequence directly (*clpC*, *dnaG*, *dnaJ*, *hsp60*, *rpoC*, *rpoB* and *purF*). This tree (Fig. S9) was reconstructed with the neighbour-joining method (Saitou & Nei, 1987), and evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980) using MEGA version 6.0 (Tamura *et al.*, 2013). The tree was rooted using *Mycobacterium tuberculosis* H37Rv^T (Fig. S9). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985). The tree topology was also confirmed by the maximum-likelihood method (Cavalli-Sforza & Edwards, 1967) using MEGA version 6.0 (Tamura *et al.*, 2013) (Fig. S10).

Estimation of the G + C content in bacterial chromosomal DNA of strain LMC 13^T was made at the DSMZ (Braunschweig, Germany). DNA was purified on hydroxyapatite according to the procedure of Cashion *et al.* (1977) and hydrolysed

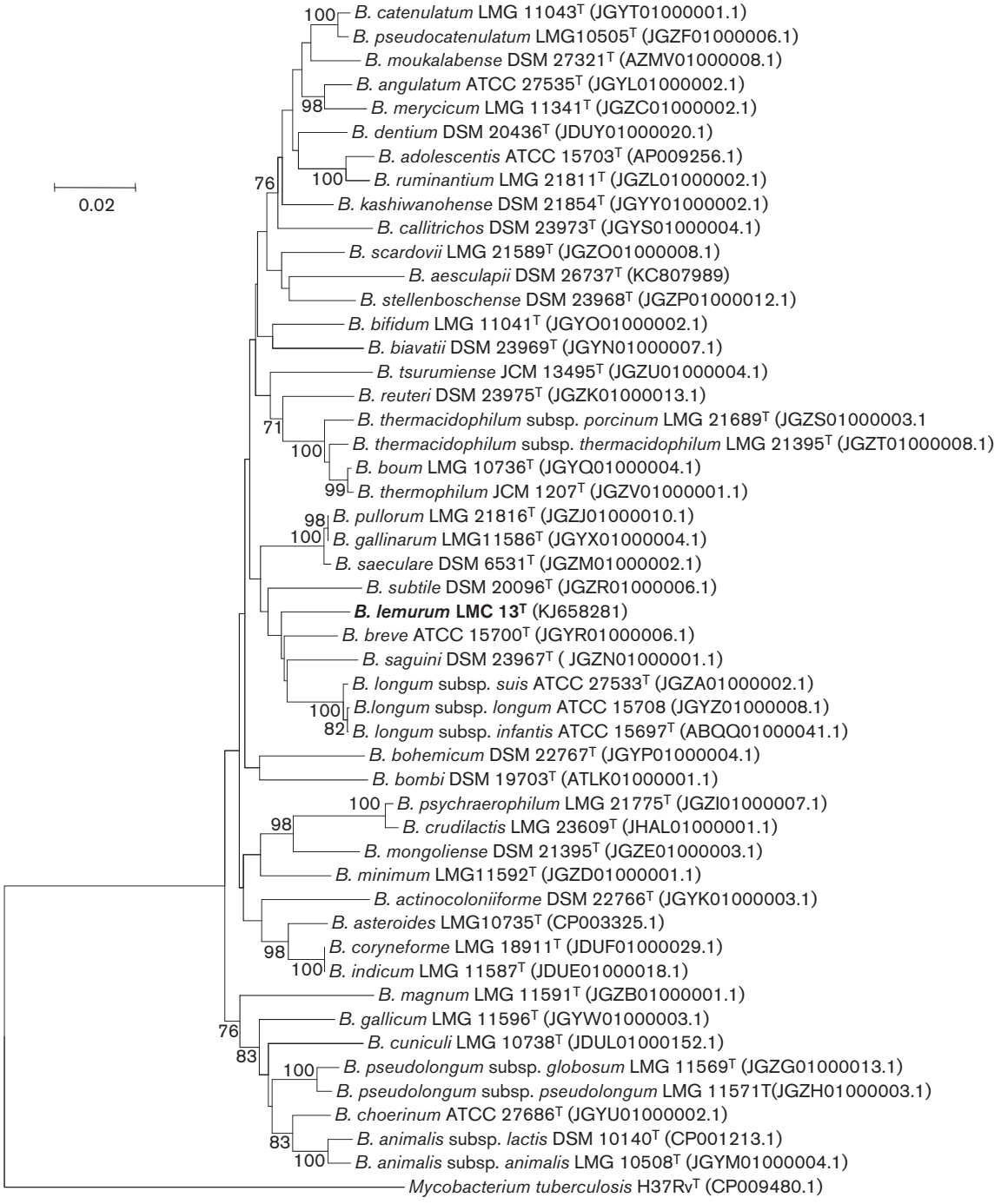

Fig. 1. Phylogenetic relationship between strain LMC 13^T and all species within the genus *Bifidobacterium* based on 16S rRNA gene sequences. The tree was reconstructed by the neighbour-joining method and rooted with *Mycobacterium tuberculosis* H37Rv^T. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; values above 70 % are given at branching points. Bar, 0.02 substitutions per nucleotide position.

enzymically by the method of Mesbah *et al.* (1989). The resulting deoxyribonucleosides were analysed by HPLC, as described by Tamaoka & Komagata (1984). Strain LMC 13^T had a DNA G+C content of 57.2 mol%. This value is within the range reported for the genus *Bifidobacterium*, 52–67 mol% (Biavati & Mattarelli, 2012; Killer *et al.*, 2010), and was very similar to that obtained recently from *B. saguini* (57.3 mol%; Endo *et al.*, 2012).

Table 1. Highest similarity between strain LMC 13^T and members of related bifidobacterial species

Related strain	Similarity to strain LMC 13^T (%)							
	16S rRNA	*hsp60*	*rpoB*	*rpoC*	*clpC*	*dnaG*	*dnaJ*	*purF*
B. longum subsp. *suis* ATCC 27533^T	96.65	92.3					78.2	87.2
B. saguini DSM 23967^T	96.64							
B. scardovii LMG 21589^T		94.4		92.4	87.0			
B. longum subsp. *longum* ATCC 15708^T		93.0					79.4	
B. reuteri DSM 23975^T		93.0						
B. longum subsp. *infantis* ATCC 15697^T		92.6			87.5	85.8	78.0	88.6
B. pullorum LMG 21816^T			93.3					
B. gallinarum LMG 11586^T			93.0					
B. cuniculi LMG 10738^T			94.0					
B. saeculare LMG 14934^T			92.7					
B. subtile LMG 11597^T				92.7		86.4		
B. breve ATCC 15700^T				92.4		86.7		87.4
B. bifidum LMG 11597^T					88.1			

Morphological, cultural and biochemical characterizations of the isolate according to standard techniques were performed at 37 °C unless otherwise stated. The morphology of cells of strain LMC 13^T, as revealed by phase-contrast microscopy, is shown in Fig. 3(a), and morphological characteristics as determined using a scanning electron microscope (SEM) are shown in Fig. 3(b, c). For SEM observations, strains were cultured on TPY agar at 37 °C for 48 h under anaerobic conditions. After culturing, a slice of agar was excised and dehydrated with a series of increasing ethanol concentrations (50, 70, 80, 90, 95 and 100 % for 15 min each). The prepared cells were subsequently critical-point-dried in a critical-point dryer apparatus (CPD Emitech K850) using liquid CO_2 as the transitional fluid. Dried samples were mounted on aluminium stubs with silver glue, and coated with gold–palladium film using an ion-sputtering unit (Emitech K500); observations were made in a Philips 515 SEM at 7–10.0 kV.

The temperature range for growth of the strain was tested using anaerobic TPY broth at 20, 25, 30, 35, 37, 40, 42, 45 and 46 °C for 48 h. The sensitivity of the strain to low pH was determined at 37 °C in anaerobic TPY broth (at pH 3.5, 4.0, 4.5, 5.0 and 5.5) for 48 h. The ability of the strain to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was tested using TPY agar, TPY soft agar (0.6 %), TPY broth, skimmed milk and UHT whole milk at 37 °C for 48 h. Haemolytic activity was determined on Columbia blood agar (Biolife) at 37 °C under anaerobic conditions for 48 h (Pineiro & Stanton, 2007). Spore staining was performed using malachite green dye. Phase-contrast microscopy (Zeiss) was used to observe the morphology of individual cells as well as spore staining.

Gram staining and catalase and oxidase activities were assessed using cells grown on TPY agar at 37 °C for 48 h under anaerobic conditions using individual Gram-staining reagents (Merck Millipore), a 3 % (v/v) hydrogen peroxide solution and cotton swabs impregnated with *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine dihydrochloride and dried (Oxibioswab; Biolife), respectively. Strain motility was determined by stabbing the culture into TPY medium containing 0.4 % agar, knowing that motile strains show a diffused growth spreading away from the line of inoculation.

Biochemical characterization was carried out using the API20A, API 20E, API ZYM and API 50CHL systems (bioMérieux), following the manufacturer's instructions with some modifications, as suggested previously (Watanabe *et al.*, 2009). Briefly, cells from agar plates were suspended in CHL broth supplemented with 0.025 % cysteine hydrochloride, inoculated into the API 50CHL test strips and incubated in an anaerobic jar at 37 °C for 5 days. The results are summarized in Table 2.

Bifidobacteria and members of related genera degrade hexoses via the fructose-6-phosphate phosphoketolase (F6PPK) pathway. The key enzyme in this pathway, F6PPK, is considered a taxonomic marker for the identification of members of *Bifidobacterium* and related genera (Biavati & Mattarelli, 2012). Detection of F6PPK activity was done according to the method described by Scardovi (1986) and modified by Orban & Patterson (2000). All the isolates possessed F6PPK activity (Table 1).

The cell-wall murein composition of strain LMC 13^T was examined by the DSMZ, using published protocols (Schumann, 2011). Analysis of partial acid hydrolysates revealed the presence of murein type A3β L-Orn–L-Ser–L-Ala–L-Thr–L-Ala. This murein type is not unique among members of the genus *Bifidobacterium*, as it has also been found in *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. longum* subsp. *suis*, suggesting the relatedness of these species.

According to phylogenetic analyses based on the 16S rRNA gene and on partial *hsp60*, *clpC*, *rpoC*, *rpoB*, *dnaG*, *dnaJ* and *purF* sequences, and other data, strain LMC 13^T is

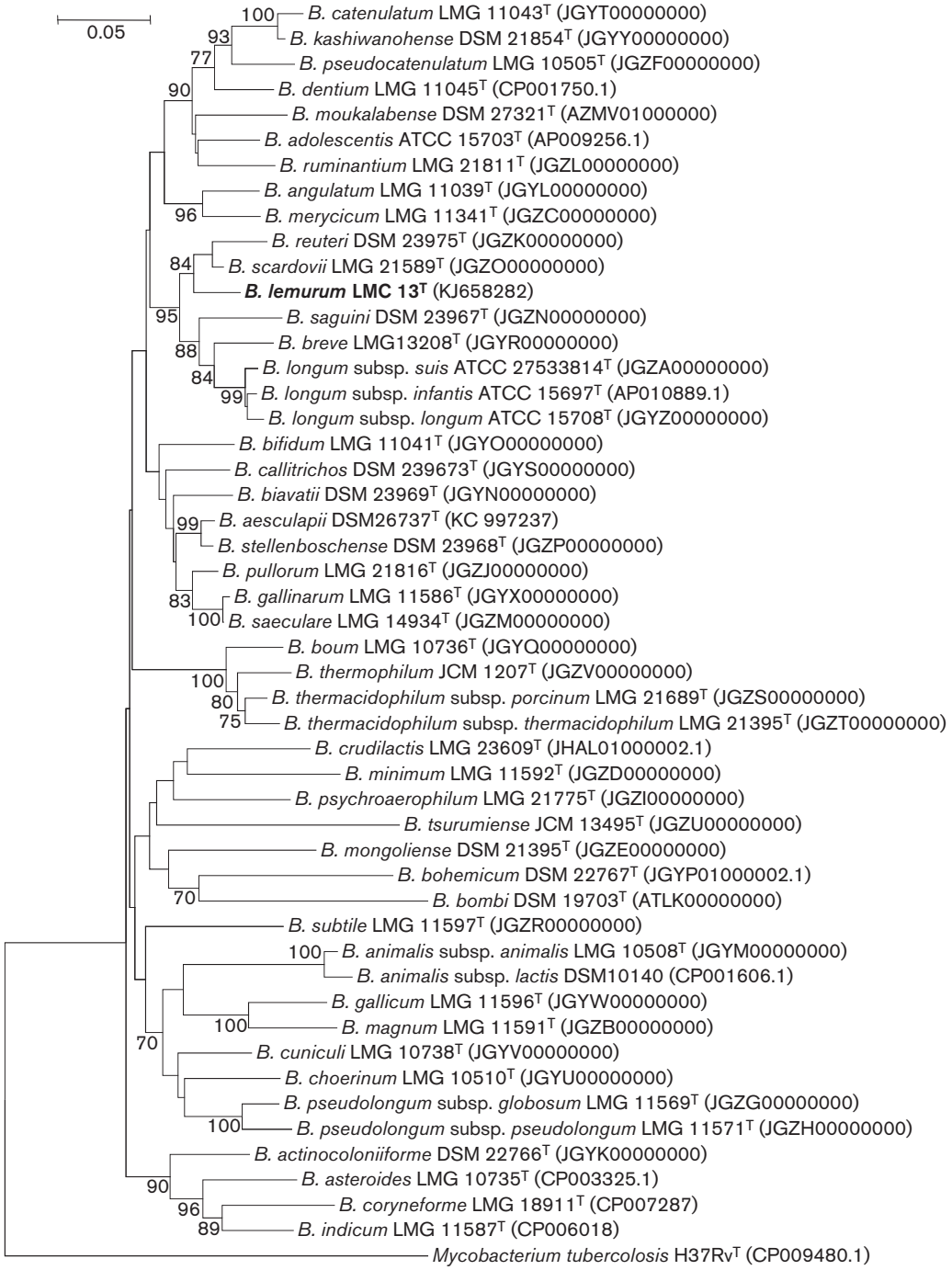

Fig. 2. Phylogenetic tree based on *hsp60* gene sequences showing the relationship between strain LMC 13^T and all the species within the genus *Bifidobacterium*. The tree was reconstructed by the neighbour-joining method and *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; values $\geqslant$70 % are given at branching points. Bar, 0.05 substitutions per nucleotide position.

genetically and phenotypically distinguishable from the currently recognized species of bifidobacteria, and thus represents a novel species, for which we propose the name *Bifidobacterium lemurum* sp. nov.

Description of *Bifidobacterium lemurum* sp. nov.

Bifidobacterium lemurum (le.mu′rum. N.L. gen. masc. pl. n. *lemurum* of/from lemurs, and the genus name of the true

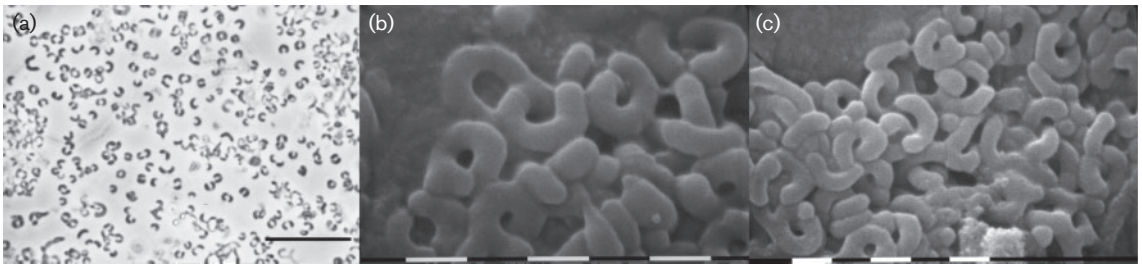

Fig. 3. Cellular morphology of strain LMC 13^T grown on TPY agar. (a) Phase-contrast photomicrograph. (b, c) Scanning electron photomicrographs. Bars, 10 μm (a) and 1 μm (b, c).

Table 2. Differential characteristics between the novel strain LMC 13^T and its closest phylogenetic relatives

Strains: 1, LMC 13^T; 2, *B. longum* subsp. *suis* ATCC 27533^T; 3, *B. saguini* DSM 23967^T; 4, *B. scardovii* LMG 21589^T. Data are from this study unless indicated. +, Positive; −, negative; W, weakly positive. All strains are negative for acid production from glycerol. All strains are positive for acid production from L-arabinose, D-galactose, D-glucose, aesculin ferric citrate, salicin, maltose, lactose (bovine origin), melibiose, raffinose and sucrose and activity of leucine arylamidase and acid phosphatase.

Characteristic	1	2	3	4
Acid production from:				
D-Arabinose	−	W	W	+
D-Ribose	+	W	+	+
D-Xylose	+	+	+	−
L-Xylose	−	W	W	−
Methyl β-D-xylopyranoside	−	−	+	−
D-Fructose	W	+	+	+
D-Mannose	+	+	W	+
L-Sorbose	−	W	W	−
D-Mannitol	W	−	−	−
Methyl α-D-mannopyranoside	−	−	W	−
Methyl α-D-glucopyranoside	W	−	+	+
N-Acetylglucosamine	−	−	W	−
Amygdalin	W	−	+	+
Arbutin	+	−	+	+
Cellobiose	+	W	+	+
Trehalose	W	−	−	+
Melezitose	+	−	−	+
DNA G+C content (mol%)	57.2	60.0[a*]	57.3[b]	60.1[c]
Enzyme activity				
Alkaline phosphatase	−	+	−	−
Esterase (C4)	−	+	+	−
Esterase lipase (C8)	−	+	−	−
Valine arylamidase	−	−	−	+
Cystine arylamidase	−	W	−	+

*Data taken from: *a*, Matteuzzi *et al.* (1971); *b*, Endo *et al.* (2012); *c*, Hoyles *et al.* (2002).

ring-tailed lemur, *Lemur catta*, referring to the primate host of the type strain).

Cells grown in TPY broth are rods, always coiled or ring shaped or having a 'Y' shape at the end. They are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and microaerophilic. Well-isolated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–3.0 mm in diameter after 3 days of incubation. The temperature range for growth is 35–46 °C; no growth occurs at 30 or 47 °C. The optimum temperature for growth is 37–42 °C. Grows at pH 5.5–7.0, with optimum growth at pH 6.5–7.0. Grows in milk under both microaerophilic and anaerobic conditions. Acid is produced from D-glucose, L-arabinose, D-ribose, D-xylose, D-galactose, D-mannose, arbutin, cellobiose, maltose, lactose, melibiose, sucrose, melezitose, raffinose, glycogen, D-mannitol, inositol, D-sorbitol, L-rhamnose, amygdalin, salicin, trehalose, potassium 2-ketogluconate and potassium 5-ketogluconate. Acid may or may not be produced from D-fructose, methyl α-D-glucopyranoside, amygdalin and turanose. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannopyranoside, *N*-acetylglucosamine, inulin, starch, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol or potassium gluconate. Results from the API ZYM test reveal production of leucine arylamidase, acid phosphatase, α- and β-galactosidases and α- and β-glucosidases. Aesculin is hydrolysed. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. longum* subgroup of the genus *Bifidobacterium*.

The type strain LMC 13^T (=JCM 30168^T=DSM 28807^T) was isolated from fresh faecal samples of an adult subject of the ring-tailed lemur (*Lemur catta*), housed in February 2014 under semi-natural conditions in Parco Natura Viva, Verona, northern Italy. The DNA G+C content of the type strain is 57.2 mol%.

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PAPER 4

Michelini, S., Oki, K., Yanokura, E., Makino, H., Shimakawa, Y., Modesto, M., Mattarelli, P., Biavati, B. & Watanabe, K. (2015). *Bifidobacterium myosotis* sp. nov., *Bifidobacterium tissieri* sp. nov and *Bifidobacterium hapali* sp. nov.: three novel taxa from the faeces of baby common marmoset (*Callithrix jacchus* L.). *International journal of systematic and evolutionary microbiology*. doi: 10.1099/ijsem.0.000708.

Bifidobacterium myosotis sp. nov., *Bifidobacterium tissieri* sp. nov. and *Bifidobacterium hapali* sp. nov., isolated from faeces of baby common marmosets (*Callithrix jacchus* L.)

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In a previous study on bifidobacterial distribution in New World monkeys, six strains belonging to the *Bifidobacteriaceae* were isolated from faecal samples of baby common marmosets (*Callithrix jacchus* L.). All the isolates were Gram-positive-staining, anaerobic, asporogenous and fructose-6-phosphate phosphoketolase-positive. Comparative analysis of 16S rRNA gene sequences revealed relatively low levels of similarity (maximum identity 96 %) to members of the genus *Bifidobacterium*, and placed the isolates in three independent clusters: strains of cluster I (MRM_5.9^T and MRM_5.10) and cluster III (MRM_5.18^T and MRM_9.02) respectively showed 96.4 and 96.7 % 16S rRNA gene sequence similarity to *Bifidobacterium callitrichos* DSM 23973^T, while strains of cluster II (MRM_8.14^T and MRM_9.14) showed 95.4 % similarity to *Bifidobacterium stellenboschense* DSM 23968^T. Phylogenetic analysis of partial *hsp60* and *clpC* gene sequences supported an independent phylogenetic position of each cluster from each other and from the related type strains *B. callitrichos* DSM 23973^T and *B. stellenboschense* DSM 23968^T. Clusters I, II and III respectively showed DNA G+C contents of 64.9–65.1, 56.4–56.7 and 63.1–63.7 mol%. The major cellular fatty acids of MRM_5.9^T were $C_{14:0}$, $C_{16:0}$ and $C_{18:1}\omega9c$ dimethylacetal, while $C_{16:0}$ was prominent in strains MRM_5.18^T and MRM_8.14^T, followed by $C_{18:1}\omega9c$ and $C_{14:0}$. Biochemical profiles and growth parameters were recorded for all the isolates. Based on the data provided, the clusters represent three novel species, for which the names *Bifidobacterium myosotis* sp. nov. (type strain MRM_5.9^T=DSM 100196^T=JCM 30796^T), *Bifidobacterium hapali* sp. nov. (type strain MRM_8.14^T=DSM 100202^T=JCM 30799^T) and *Bifidobacterium tissieri* sp. nov. (type strain MRM_5.18^T=DSM 100201^T=JCM 30798^T) are proposed.

†These authors contributed equally to this work.

Abbreviations: DDH, DNA–DNA hybridization; F6PPK, fructose-6-phosphate phosphoketolase.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *hsp60* and *clpC* gene sequences of *B. myosotis* sp. nov. MRM_5.9^T and MRM_5.10 are KP718941 and KP718942, KP732524 and KP732525, and KP732530 and KP732531, respectively; those of *B. tissieri* MRM_5.18^T and MRM_9.02 are KP718951 and KP718957, KP732526 and KP732528, and KP732532 and KP732534, respectively; and those of *B. hapali* MRM_8.14^T and MRM_9.14 are KP718961 and KP718963, KP732527 and KP732529, and KP732533 and KP732535, respectively.

A supplementary figure and four supplementary tables are available with the online Supplementary Material.

Bifidobacteria are Gram-positive, anaerobic, non-motile and non-spore-forming bacteria, and are generally host-animal-specific, being separated into 'human' and 'animal' groups on the basis of their distribution (Ventura *et al.*, 2004). Members of the genus *Bifidobacterium* are typically found in the gastrointestinal tracts of humans and other mammals, but also in the hindgut of important pollinators (honeybees, bumblebees, etc.) (Biavati & Mattarelli, 2012; Killer *et al.*, 2009; Kopečný *et al.*, 2010). They have also been isolated from waste and dairy products, where the sources could respectively be faecal contamination and intentional probiotic addition (Mattarelli & Biavati, 2014). The family *Bifidobacteriaceae* currently consists of nine genera with validly published names: *Alloscardovia*, *Aeriscardovia*, *Bifidobacterium*, *Gardnerella*, *Metascardovia*, *Parascardovia*, *Scardovia* (Biavati & Mattarelli, 2012), *Bombiscardovia* (Killer *et al.*, 2010) and *Pseudoscardovia* (Killer *et al.*, 2013). At the time of writing, the genus *Bifidobacterium* comprised 45 species and nine subspecies with validly published names.

The present work deals with bifidobacteria isolated from common marmosets (*Callithrix jacchus* L.), small New-World monkeys that eat plant exudates and insects and that have developed a large specialized caecum for the digestion of the complex carbohydrates found in tree exudates (Bailey & Coe, 2002; Caton *et al.*, 1996). In a previous work, Michelini *et al.* (2015) explored bifidobacterial distribution in baby common marmosets. A total of 92 bifidobacterial strains were isolated from samples of fresh rectal swabs from five individuals. Band profiling and clustering analysis, by BOX-PCR and *hsp60* RFLP-PCR, highlighted the presence of three known species, *Bifidobacterium aesculapii*, *B. callitrichos* and *B. reuteri*, and five putative novel bifidobacterial species (Michelini *et al.*, 2015). In the present study, selected representative strains belonging to three of the five previously detected putative novel species, strains MRM_5.9^T and MRM_5.10 (forming cluster I), MRM_8.14^T and MRM_9.14 (cluster II) and MRM_5.18^T and MRM_9.02 (cluster III), were characterized in detail.

Subculturing and maintenance of cultures was performed according to Modesto *et al.* (2014). In all experiments (unless otherwise specified), the strains were cultivated in TPY broth at pH 6.9 and 37 °C under anaerobic conditions. Morphological and biochemical profiles, growth parameters and molecular characterization were evaluated for the selected strains.

Chromosomal DNA was extracted according to the method of Marmur (1961). The DNA concentration was determined spectrophotometrically from the A_{260}, and the purity of each sample was estimated by determining the A_{260}/A_{280} ratio.

The conditions for PCR amplification of the partial 16S rRNA gene, and subsequent DNA sequencing, were described previously (Chao *et al.*, 2008). Conditions for amplification and sequencing of the partial *hsp60* gene were described by Baffoni *et al.* (2013); primers H60F (5′-GGNGAYGGNACNACNACNGCNACNGT-3′) and H60R (5′-TCNCCRAANCCNGGNGCYTTNACNGC-3′) were used. PCR amplification was performed using 25 µl of a mixture containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 200 mM of each dNTP, 0.4 µM of each primer, 1 U *Taq* polymerase (Takara Bio) and 10 ng template DNA. The amplification program for the *hsp60* gene consisted of one cycle of 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 61 °C for 1 min and 72 °C for 45 s, and a final amplification step at 72 °C for 10 min. The conditions for PCR amplification of the partial *clpC* gene have been described by Yanokura *et al.* (2015), using primers clpC-F (5′-GAGTACCGCAAGTACATCGAG-3′) and clpC-R (5′-TCATCGTCGAACAGGAAC-3′). The amplification program for the *clpC* gene consisted of one cycle of 94 °C for 2 min, 32 cycles of 94 °C for 20 s, 51 °C for 20 s and 72 °C for 20 s, and a final amplification step at 72 °C for 3 min. The PCR-amplified 16S rRNA, *hsp60* and *clpC* genes were purified according to Yanokura *et al.* (2015).

The partial 16S rRNA, *hsp60* and *clpC* gene sequences of the six strains and of the type strains of closely related species retrieved from the DDBJ/GenBank/EMBL databases were aligned with CLUSTAL Omega in CLC Sequence Viewer version 7.5 (www.clcbio.com). The Gblocks program (version 0.91b), a server tool at the Castresana laboratory (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was then used to eliminate poorly aligned positions and divergent regions of DNA alignments, facilitating phylogenetic analysis (Talavera & Castresana. 2007).

Phylogenetic trees, based on partial 16S rRNA, *hsp60* and *clpC* gene sequences of all 51 taxa of the genus *Bifidobacterium* with validly published names, were reconstructed with the neighbour-joining method (Saitou & Nei, 1987); evolutionary distances were computed using Kimura's two-parameter method and are in units of the number of base substitutions per site. Bootstrap analysis of 1000 replicates (Felsenstein, 1985) was performed to evaluate the statistical reliability of the trees. Any positions with less than 97 % site coverage were eliminated (Figs. 1–3). *Micrococcus luteus* DSM 20030^T was used as the outgroup for 16S rRNA and *clpC* tree reconstruction, and *Mycobacterium tuberculosis* ATCC 27294^T was used as an outgroup for the *hsp60* tree. The 16S rRNA tree topology was also confirmed with the maximum-likelihood method (Cavalli-Sforza & Edwards, 1967) in MEGA6 (Tamura *et al.*, 2013), using the same parameters (Fig. S1, available in the online Supplementary Material).

Similarity/identity matrices of the 16S rRNA gene, *hsp60* and *clpC* gene sequences were calculated using MatGat version 2.03 (Campanella *et al.*, 2003). Based on these results, three independent clusters were confirmed: strains MRM_5.9^T and MRM_5.10 (cluster I), MRM_8.14^T and MRM_9.14 (cluster II) and MRM_5.18^T and MRM_9.02. (cluster III). Strains of each cluster shared low sequence similarity (74.2–96.7 %) with each other and with bifidobacterial taxa with validly published names. Only the

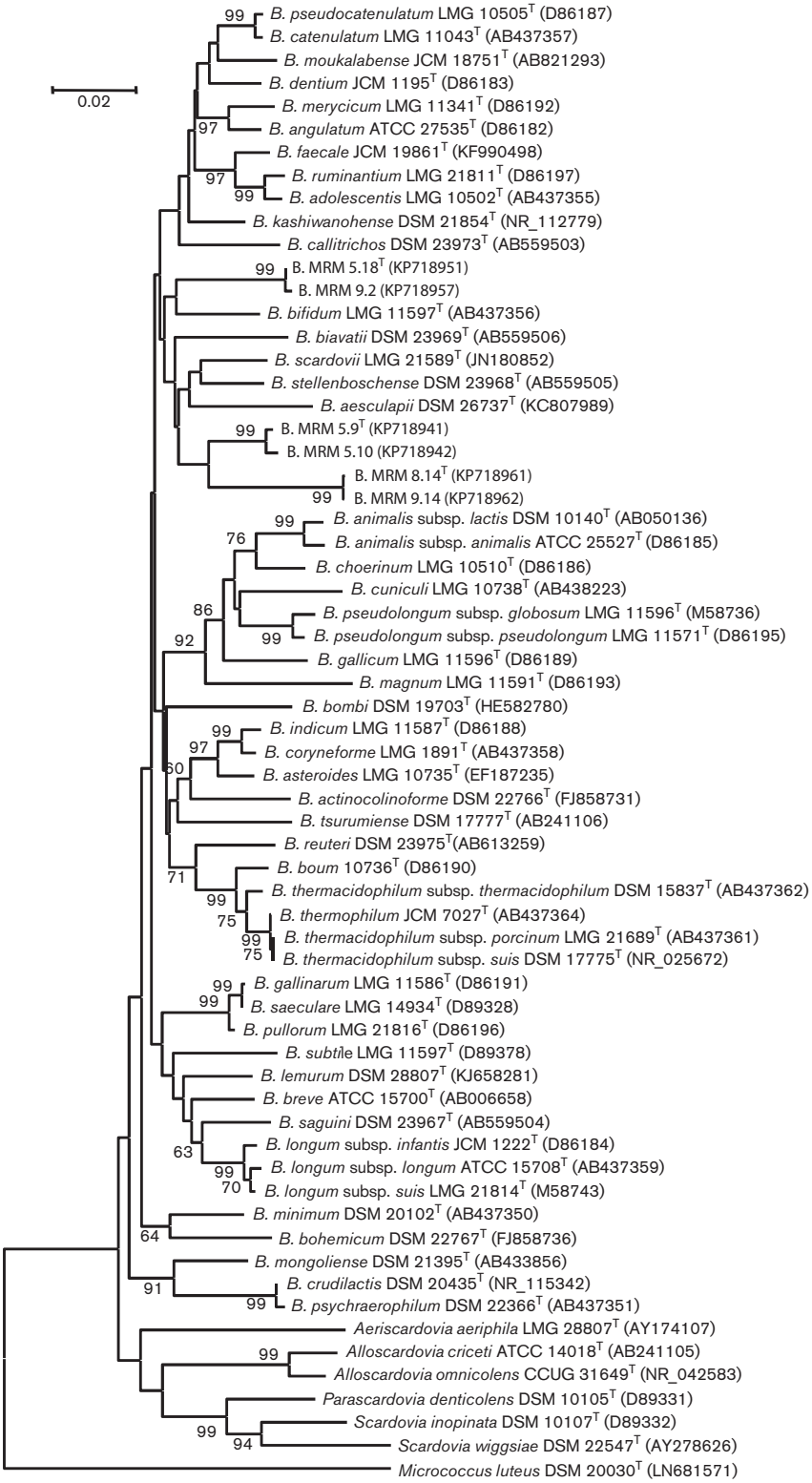

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of the novel strains isolated from baby common marmosets with all species of *Bifidobacterium* and members of related genera of the *Bifidobacteriaceae*. The tree was reconstructed by the neighbour-joining method and *Micrococcus luteus* DSM 20030^T was used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; values above 60 % are shown. Bar, 0.02 substitutions per nucleotide position.

strains of cluster I showed high *hsp60* gene sequence similarity (ranging from 98.6–99.4 %), to the type strain of *Bifidobacterium lemurum*, a species recently described from *Lemur catta* (Modesto *et al.*, 2015). Phylogenetic relatives are shown in Table S1.

Based on the neighbour-joining analysis of the 16S rRNA gene sequences, the strains of the first and second clusters are phylogenetically related, composing two subgroups; the third cluster is closely related to the others, but it forms a separate group (Fig. 1). For the *clpC* and *hsp60* phylogenetic trees, we recognized a topological variation among the sequences of the three clusters (Figs. 2 and 3). However, many factors, such as the stochastic nature of mutation, lineage sorting and phylogenetic reconstruction artefacts, could cause different genes to give different topologies (Castresana, 2007).

An additional tree was created on the basis of the concatenation of the 16S rRNA, *rpoB* and *clpC* gene sequences. This tree (Fig. S2) was reconstructed with the neighbour-joining method (Saitou & Nei, 1987), and evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980) using MEGA version 6.0 (Tamura *et al.*, 2013). The tree was rooted using *Mycobacterium tuberculosis* H37Rv^T. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985). The tree topology was also confirmed by the maximum-likelihood method (Cavalli-Sforza & Edwards, 1967) using MEGA version 6.0 (Tamura *et al.*, 2013) (Fig. S3).

16S rRNA gene sequence similarity below 97 % to the most closely related species supports the establishment of a novel species (Tindall *et al.*, 2010). To support the identification of novel bifidobacterial taxa, we carried out a DNA–DNA hybridization (DDH) analysis of the isolated strains, both between the strains themselves and with the related type strains *B. callitrichos* DSM 23973^T and *B. stellenboschense* DSM 23968^T. DDH analysis was performed according to the microdilution well technique, using photobiotin for DNA labelling (Ezaki *et al.*, 1989). Reciprocal DDH experiments were performed for each pair of strains at 55 °C for 2 h in the presence of 50 % formamide. After 30 min of incubation at 37 °C with the addition of 4-methylumbelliferyl β-D-glucopyranoside solution, fluorescence intensity was measured. The data were calculated as the mean taken by excluding the highest and lowest values of eight replicate wells for each experiment.

DNA–DNA relatedness among strains in the same cluster and their phylogenetic neighbours was evaluated. The results, summarized in Table S2, confirm the identification of three independent taxa. DDH between the two strains of cluster I (MRM_5.9^T and MRM_5.10) and the related type strain *B. callitrichos* DSM 23973^T was 34.7 and 35.5 %, respectively, below the 70 % recommended cut-off value for species demarcation (Wayne *et al.*, 1987). The strains of cluster II (MRM_8.14^T and MRM_9.14) had low DDH to the related type strain *B. stellenboschense* DSM 23968^T, 3.8 and 10.3 %, respectively, whereas the strains of cluster III (MRM_5.18^T and MRM_9.02) showed DDH to *B. callitrichos* DSM 23973^T of 26.8 and 39.7 %, respectively.

An estimation was made of the chromosomal DNA G+C content of strains of each cluster, for both the type and reference strains. After extraction, the DNA was degraded enzymically into nucleosides and then separated by HPLC, as described previously (Mesbah *et al.*, 1989). The DNA G+C content of the strains of clusters I and III was 64.9, 65.1, 63.1 and 63.7 mol%, while lower values, 56.4 and 56.7 mol%, were found for the strains of cluster II (Table S2).

A phenotypic characterization of the six strains was performed. The morphologies, examined by phase-contrast microscopy, are shown in Fig. 4. The novel isolates and related type strains, *B. stellenboschense* DSM 23968^T, *B. callitrichos* DSM 23973^T and *Bifidobacterium bifidum* LMG 11597^T, were also investigated for substrate utilization and enzyme production, using API 50 CHL and Rapid ID 32 test kits (bioMérieux) (Table 1).

Optimal growth conditions were determined in TPY broth after 24 h of incubation under anaerobic conditions. The tested temperatures were 20, 25, 30, 35, 37, 40, 42, 45 and 47 °C. Growth at low pH was screened at pH 3.5, 4.0, 4.5, 5.0 and 5.5. The ability of the strains to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was also tested on TPY agar and in TPY broth after 48 h of incubation at 37 °C (Table 1).

Haemolytic activity was determined for 48 h in Columbia blood agar (Biolife), at 37 °C under anaerobic conditions (Pineiro & Stanton, 2007).

Gram staining of each strain was carried out on cells grown on TPY agar for 48 h at 37 °C under anaerobic conditions, and using individual Gram staining reagents (Merck Millipore). Catalase and oxidase activities were assessed according to Modesto *et al.* (2014).

Cellular fatty acid methyl esters were obtained from cells grown in GAM broth (Nissui Pharmaceutical) with 0.5 % glucose at 37 °C for 1 day by saponification, methylation and extraction, using the method of Miller (1982) with minor modifications (Kuykendall *et al.*, 1988). The cellular fatty acid profiles for each strain and type strains of related species are shown in Table 2. Palmitic, myristic and oleic acids, the dominant fatty acids, were detected in all the clusters but in different amounts. Notably, the strains of cluster III showed the highest levels of palmitic acid ($C_{16:0}$) (mean 30.33 %) and oleic acid ($C_{18:1}\omega 9c$) (mean 14.63 %). Stearic acid ($C_{18:0}$) was found only in clusters I and III and in *B. stellenboschense* DSM 23968^T, whereas capric acid ($C_{10:0}$) characterized the profile of cluster I. Moreover, two fatty acids showed a strain-specific distribution: undecyclic acid ($C_{11:0}$) was detected in strain MRM_9.14 but not in strain MRM_8.14^T of cluster II, and $C_{18:1}\omega 6c$ was present in the profile of strain MRM_5.9^T but not in MRM_5.10 of cluster I.

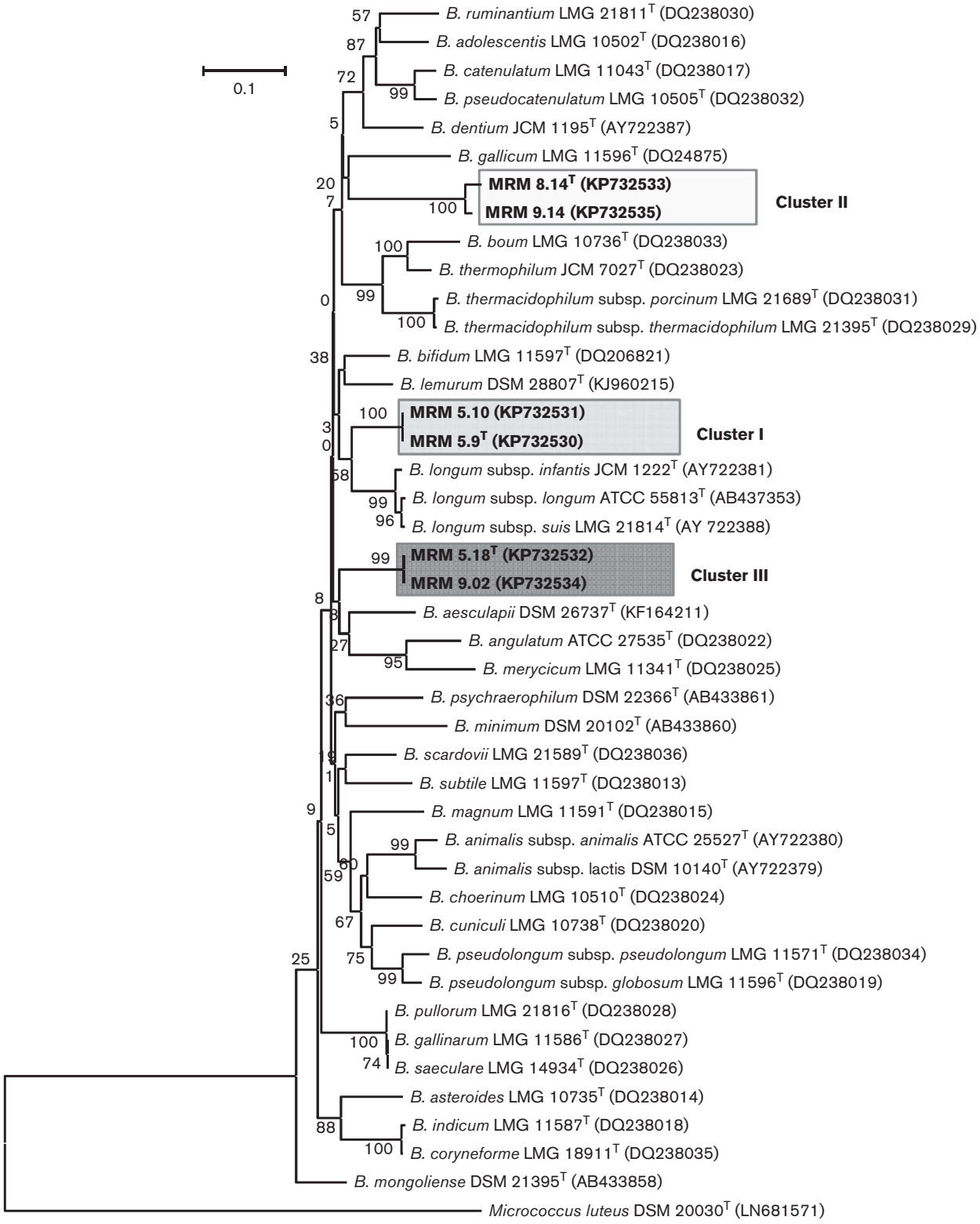

Fig. 2. Phylogenetic tree based on *clpC* gene sequences showing the relationships of the novel strains isolated from baby common marmosets with all species of *Bifidobacterium*. The tree was reconstructed by the neighbour-joining method and *Micrococcus luteus* DSM 20030^T was used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches; values above 70 % are shown. Bar, 0.1 substitutions per nucleotide position.

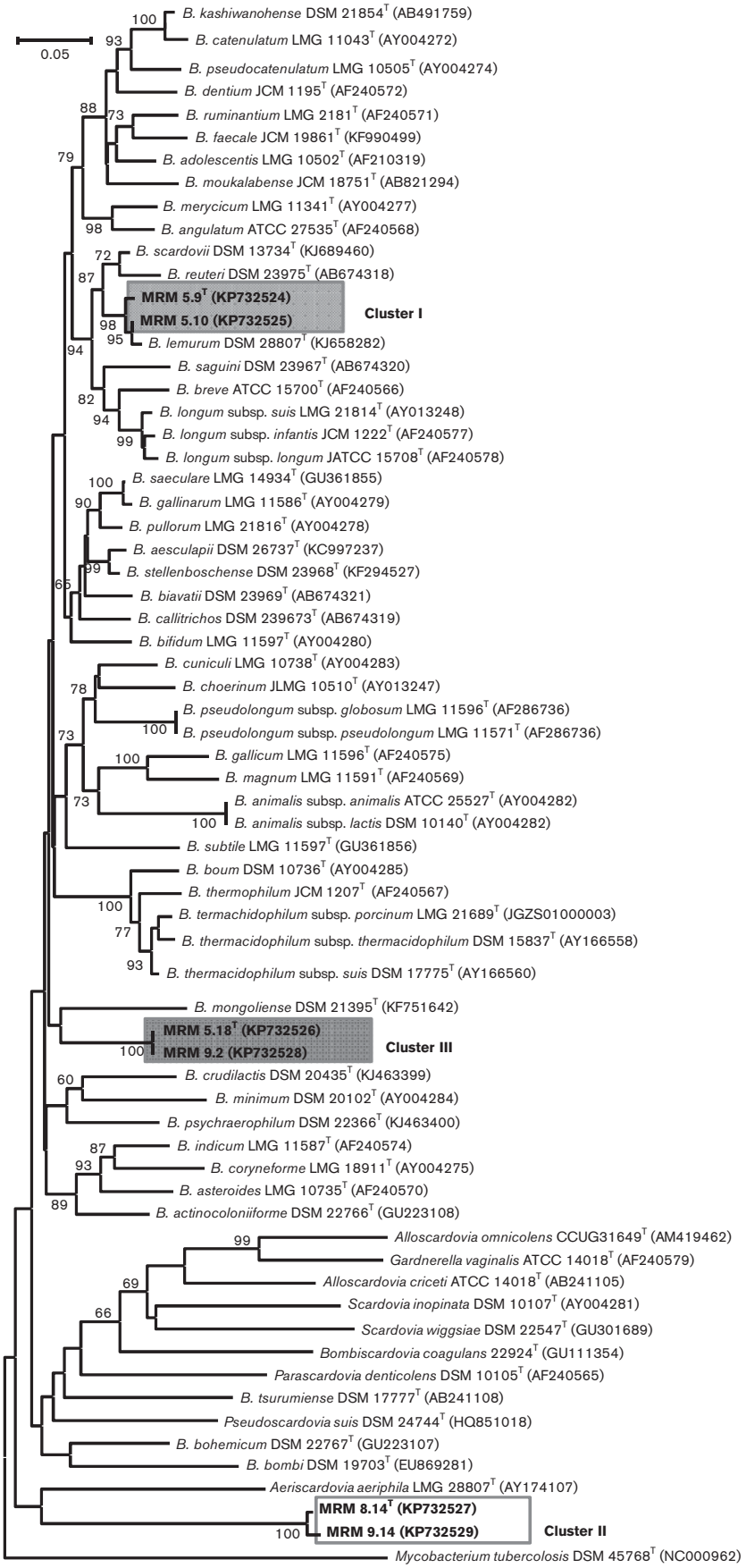

Fig. 3. Phylogenetic tree based on *hsp60* gene sequences showing the relationships of the novel strains isolated from baby common marmosets with all species of *Bifidobacterium*. The tree was reconstructed by the neighbour-joining method and *Mycobacterium tuberculosis* DSM 45768^T was used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches; values over 60 % are shown. Bar, 0.05 substitutions per nucleotide position.

The cell-wall peptidoglycan composition of the strains was examined. Cell walls were prepared and hydrolysed according to the method of Schleifer & Kandler (1972). The cell-wall amino acids were analysed by HPLC (Alliance 2695 HPLC system; Waters) equipped with a fluorescence detector (model 474; Waters) and AccQ-Tag column (3.9 × 150 mm; Waters), and using an AccQ-Fluor reagent kit (6-aminoquinolyl *N*-hydroxysuccinimidylcarbamate; Waters) for derivatization. The peptidoglycan type in clusters I (MRM_5.9^T and MRM_5.10) and III (MRM_5.18^T and MRM_9.02) was L-Glu–L-Ala–L-Lys, with the presence of Ala, Glu and Lys (ratio 1.0 : 1.0 : 0.7), and, in cluster II (MRM_9.14^T and MRM_8.14), it was L-Lys (Orn)–L-Ala$_2$–L-Ser, with the presence of Ala, Lys + Orn, Ser and Glu (ratio 3 : 2 : 1 : 1) (Table S3).

Fructose-6-phosphate phosphoketolase (F6PPK) is the key enzyme involved in the degradation of hexoses in the F6PPK pathway, and represents a taxonomic marker in identification of the genus *Bifidobacterium* (Biavati & Mattarelli, 2012). The phosphoketolase test for the identification of bifidobacteria, developed by Scardovi (1986) and modified by Orban & Patterson (2000), was performed. All the isolates were positive for the presence of the enzyme.

Metabolic end products from glucose were analysed by ion-exclusion HPLC, as described by Kikuchi & Yajima (1992). Briefly, a mixture of 0.9 ml supernatant of a 1 day culture in modified GAM broth (supplemented with 1 % glucose) and 0.1 ml 10 % (v/v) $HClO_4$ was allowed to stand for 4 h at 4 °C. Filtered samples with a filter of 0.45 μm pore size (Millipore) were analysed for organic acids using an HPLC-equipped Shodex RSKC-81 column (Showa Denko Co.) and a conductometric detector (model 432; Waters). There was no evidence of the production of the volatile fatty acids propionic, isobutyric, butyric, isovaleric or valeric acid as metabolic end products from glucose in any of the isolates or related species (Table S4). The strains of cluster I (MRM_5.9^T and MRM_5.10) showed the highest production of lactic and acetic acids compared with the related type strain *B. callitrichos* DSM 23973^T and with other clusters. The presence of the volatile fatty acids succinic, lactic, formic and acetic acids in strains belonging to cluster II (MRM_8.14^T and MRM_9.14) was generally lower than in the related type strain *B. stellenboschense* DSM 23968^T. Isolates from cluster III (MRM_5.18^T and MRM_9.02) produced more formic acid than did the related type strain *B. callitrichos* DSM 23973^T. The relationship between the levels of lactic and acetic acids was also calculated; all strains showed a ratio of 1 : 3.

Based on phylogenetic analyses of the partial 16S rRNA, *hsp60* and *clpC* gene sequences, and according to other data, including DDH, DNA G + C content and peptidoglycan cell-wall composition, the six isolates were genetically and phenotypically distinguishable from the currently recognized species of bifidobacteria, and represent three novel species, for which the names *Bifidobacterium myosotis* sp. nov. (cluster I, strains MRM_5.9^T and MRM_5.10), *Bifidobacterium hapali* sp. nov. (cluster II, strains MRM_8.14^T and MRM_9.14) and *Bifidobacterium tissieri* sp. nov. (cluster III, strains MRM_5.18^T and MRM_9.02) are proposed.

Description of *Bifidobacterium myosotis* sp. nov.

Bifidobacterium myosotis (my.o.so′tis. Gr. masc. n. *mys*, *myos* mouse; Gr. neut. n. *ous*, *otos* ear; N.L. n. *myosotis* a mouse ear, referring to the cell shape in live observations).

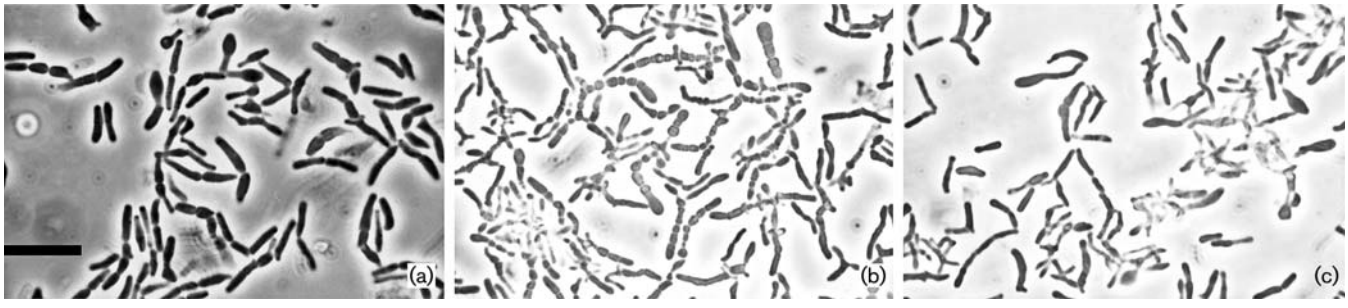

Fig. 4. Phase-contrast photomicrographs of cells of *B. myosotis* sp. nov. MRM 5.9^T (a), *B. hapali* sp. nov. MRM 8.14^T (b) and *B. tissieri* sp. nov. MRM 5.18^T (c) grown in TPY broth showing cellular morphology. Bar, 10 μm.

Table 1. Phenotypic information for representative strains of each profile type

Strains: 1, *B. myosotis* sp. nov. MRM_5.9^T; 2, *B. myosotis* sp. nov. MRM_5.10; 3, *B. tissieri* sp. nov. MRM_5.18^T; 4, *B. tissieri* sp. nov. MRM_9.02; 5, *B. callitrichos* DSM 23973^T; 6, *B. hapali* sp. nov. MRM_8.14^T; 7, *B. hapali* sp. nov. MRM_9.14; 8, *B. stellenboschense* DSM 23968^T; 9, *B. bifidum* DSM 29521^T. All data are from this study. Fermentation profiles and enzymic information were obtained from the API 50 CHL and Rapid ID32 systems under optimal growth conditions. +, Positive; −, negative; W, weakly positive; ND, not determined.

Property	1	2	3	4	5	6	7	8	9
Carbohydrate utilization									
Glycerol	−	−	+	+	−	+	−	+	+
D-Arabinose	−	−	−	−	−	−	−	−	−
L-Arabinose	+	+	+	W	+	+	+	+	−
D-Ribose	−	−	−	−	−	−	−	+	−
D-Xylose	+	+	+	W	+	W	+	+	−
Methyl β-D-xylopyranoside	−	+	−	−	−	+	−	−	−
D-Galactose	−	+	+	W	+	−	−	+	+
D-Glucose	+	+	+	+	−	+	+	−	+
D-Fructose	−	+	+	+	−	+	+	+	−
D-Mannose	W	+	+	W	+	−	+	−	−
L-Rhamnose	−	−	+	W	−	−	−	−	−
D-Mannitol	−	W	W	W	+	−	−	+	−
D-Sorbitol	−	−	−	−	−	−	−	+	−
Methyl α-D-glucopyranoside	−	−	+	+	−	−	−	+	−
N-Acetylglucosamine	−	−	W	−	−	−	−	+	−
Amygdalin	−	W	−	−	−	−	−	+	−
Arbutin	−	−	−	−	−	−	−	+	−
Salicin	+	+	+	+	+	W	+	+	−
Cellobiose	−	+	+	+	+	+	W	−	−
Lactose	+	+	+	+	−	+	W	−	−
Melibiose	+	−	−	−	−	−	−	+	−
Sucrose	+	+	+	+	−	+	W	+	+
Trehalose	+	−	+	+	+	−	−	−	−
Melezitose	W	−	W	+	+	W	−	+	−
Raffinose	−	+	+	+	+	+	+	+	−
Starch	−	−	−	−	+	−	−	−	−
Gentiobiose	−	W	−	−	−	−	−	W	−
Turanose	−	W	−	−	−	−	−	+	−
Gluconate	−	−	−	−	−	−	−	+	−
2-Ketogluconate	−	−	+	W	−	−	−	−	−
Aesculin hydrolysis	+	+	+	+	−	+	+	−	−
Arginine dihydrolase	+	+	+	+	+	+	−	−	W
α-Galactosidase	−	−	−	−	−	−	W	W	−
β-Galactosidase	−	−	−	−	−	−	−	W	+
α-Glucosidase	−	−	−	−	−	−	W	W	−
β-Glucosidase	−	−	−	−	−	−	+	−	−
α-Arabinosidase	−	−	−	−	−	−	+	+	−
β-Glucuronidase	−	−	−	−	−	−	−	+	−
N-Acetyl-β-glucosaminidase	−	−	−	−	−	−	−	−	+
Glutamic acid decarboxylase	−	−	−	−	−	−	−	+	−
Alkaline phosphatase	−	−	+	+	+	+	+	+	+
Arginine arylamidase	−	−	+	+	+	+	+	+	+
Leucyl-glycine arylamidase	+	+	+	−	+	+	+	+	+
Pyroglutamic acid arylamidase	−	−	−	−	+	+	+	+	+
Glutamyl-glutamic acid arylamidase	−	−	−	−	−	+	+	+	+
Serine arylamidase	+	+	−	−	+	+	+	+	+
Temperature for growth (°C) Range	25–46	25–46	20–44	20–44	ND	25–42	25–42	ND	ND
Optimum	40	40	35	35	ND	37	37	ND	ND
pH for growth Range	4.5–8.0	4.5–8.0	5.5–7.5	5.5–7.5	ND	4.5–7.5	4.5–7.5	ND	ND
Optimum	7	7	6.5	6.5	ND	6.5	6.5	ND	ND

Table 2. Cellular fatty acid compositions of the novel strains and related type strains

Strains: 1, *B. myosotis* MRM_5.9^T; 2, *B. myosotis* MRM_5.10; 3, *B. tissieri* MRM_5.18^T; 4, *B. tissieri* MRM_9.02; 5, *B. hapali* MRM_8.14^T; 6, *B. hapali* MRM_9.14; 7, *B. callitrichos* DSM 23973^T; 8, *B. stellenboschense* DSM 23968^T. Data are from this study. Values are percentages of total fatty acids. DMA, Dimethylacetal; -: lower than detection limit.

Fatty acid	1	2	3	4	5	6	7	8
$C_{10:0}$	1.26	1.23	–	–	–	–	–	–
$C_{12:0}$	4.26	3.18	1.62	1.78	1.68	2.36	2.80	1.78
$C_{11:0}$ DMA	2.26	2.13	–	–	–	2.08	1.94	–
Summed feature 1*	1.55	1.95	1.01	1.41	1.65	1.92	2.73	1.43
$C_{14:0}$	27.22	25.02	15.26	16.51	9.25	12.87	16.74	12.63
$C_{14:0}$ DMA	10.33	13.17	6.91	8.91	7.21	11.56	13.47	7.45
$C_{16:1}\omega 9c$	4.68	3.83	2.74	2.61	1.41	1.84	3.64	1.80
$C_{16:0}$	17.48	20.44	30.49	30.16	23.13	24.25	23.63	23.60
$C_{16:0}$ DMA	–	–	–	–	1.53	1.14	–	1.40
Summed feature 7*	2.33	2.18	1.65	2.73	10.00	4.55	4.76	6.20
anteiso-$C_{17:0}$ DMA	–	–	–	–	–	–	4.74	–
$C_{18:1}\omega 9c$	7.52	7.91	16.85	12.40	9.38	7.89	8.20	9.98
Summed feature 10*	–	1.57	–	–	–	–	–	2.27
$C_{18:1}\omega 6c$	1.80	–	–	–	2.49	2.27	–	–
$C_{18:0}$	–	–	1.80	1.18	1.61	1.03	–	1.69
$C_{18:1}\omega 9c$ DMA	11.17	10.55	11.60	12.09	23.07	19.34	13.34	19.77
Summed feature 12*	–	–	–	–	–	–	–	1.04
$C_{19:0}$ cyclo 9,10	2.38	1.81	2.58	1.80	1.14	1.00	–	1.56
$C_{19:0}$ cyclo 9,10 DMA	2.75	1.69	2.12	2.43	2.39	2.37	1.34	1.93
$C_{18:0}$ 2-OH	–	–	–	–	–	–	–	1.00

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 1 contained one or more of $C_{13:1}$ at 12–13, $C_{14:0}$ aldehyde and $C_{11:1}$ 2-OH. Summed feature 7 contained $C_{17:2}$ at 16.760 and/or $C_{17:1}\omega 9c$. Summed feature 10 contained $C_{18:1}\omega 7c$ and/or unknown ECL 17.834. Summed feature 12 contained unknown ECL 18.622 and/or iso-$C_{19:0}$.

Cells grown in TPY broth are rods of varying shapes, forming a branched structure with a 'Y' at the end. The well-isolated colonies grown on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while the embedded colonies are lens-shaped or elliptical. The colonies reach a diameter of 1.0–2.0 mm after 3 days of incubation. Cells are Gram-positive-staining, non-motile, asporogenous and non-haemolytic. F6PPK-positive, catalase- and oxidase-negative, indole-negative and are able to survive under microaerophilic conditions. The temperature for growth is 25–46 °C; no growth occurs 20 or 48 °C. Cells grow at pH 4.5–8. The optimal conditions for growth are pH 7 and 40 °C. Acid is produced from L-arabinose, D-xylose, D-glucose, salicin, cellobiose, maltose, lactose, sucrose, melibiose and trehalose. Acid may or may not be produced from melezitose, D-mannose, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannitol, gentiobiose, turanose and amygdalin. Acid is not produced from glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, trehalose, inulin, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate. Aesculin is hydrolysed and urease is produced. Results from the API RAPID ID 32A test reveal production of arginine dihydrolase, proline arylamidase, leucyl-glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Alkaline phosphatase, esterase lipase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase, urease, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, β-glucosidase, α-arabinosidase and *N*-acetyl-β-glucosaminidase are not produced. Negative for reduction of nitrates, indole production and activities of alkaline phosphatase, arginine arylamidase and glutamylglutamic acid arylamidase. The major cellular fatty acids are $C_{14:0}$, $C_{16:0}$ and $C_{18:1}\omega 9c$ dimethylacetal. The peptidoglycan type is L-Glu–L-Ala–L-Lys.

The type strain, MRM_5.9^T (=DSM 100196^T=JCM 30796^T), and the reference strain MRM_5.10 (=DSM 100217=JCM 30897) were isolated from the faeces of common marmosets. The DNA G+C content of the type strain is 65.1 mol%.

Description of *Bifidobacterium hapali* sp. nov.

Bifidobacterium hapali (ha′pa.li. N.L. gen. n. *hapali* of/from *Hapale*, the original genus name of the common marmoset, *Callithrix jacchus*, from which the first strains were isolated).

Cells growing in TPY broth under anaerobic conditions are rods of various shapes, forming a branched structure with a 'Y' at the end. Well-isolated colonies on the surface of TPY agar plates reach a diameter of about 1.5–2.5 mm after 2 days of incubation under anaerobic conditions. The colonies are white, opaque, smooth and circular with entire edges, but when embedded, the colonies are lens-shaped or elliptical. Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and are able to survive under microaerophilic conditions. The temperature for growth is 25–42 °C; no growth at 20 or 44 °C. Cells grow at pH 4.5–7.5. The optimal growth conditions are pH 6.5 and 37 °C. Acid is produced from D-glucose, D-fructose, maltose, raffinose, glycerol, methyl β-D-xylopyranoside, cellobiose, lactose, sucrose and D-mannose. Acid may be or may not be produced from D-xylose, salicin and melezitose. Acid is not produced from erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, melibiose, trehalose, inulin, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, D- or L-arabitol, gluconate or 2-or 5-ketogluconate. Hydrolyses aesculin and produces urease. In tests with the API Rapid ID32A system, positive for arginine dihydrolase, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl-glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase and glutamyl-glutamic acid arylamidase. Lipase, valine arylamidase, cystine arylamidase, α-chymotrypsin, urease, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, glutamic acid decarboxylase and α-fucosidase are not produced; nitrate reduction and indole production are not detected. The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{14:0}$. The peptidoglycan type is L-Lys (Orn)–L-Ala_2–L-Ser.

The type strain, MRM_8.14^T (=DSM 100202^T=JCM 30799^T), and the reference strain MRM_9.14 (=DSM 100218=JCM 30800) were isolated from the faeces of common marmosets. The DNA G+C content of the type strain is 56.4 mol%.

Description of *Bifidobacterium tissieri* sp. nov.

Bifidobacterium tissieri [tis.si.e′ri. N.L. masc. gen. n. *tissieri* of Tissier, named after Professor Henry Tissier, a French paediatrician at the Pasteur Institute (Paris), who first isolated a bacterium characterized by a Y-shaped morphology, 'bifid', from the intestinal flora of breast-fed infants; he named the bacterium 'bifidus'].

Cells grown in TPY broth under anaerobic conditions are rods of varying shape that form a branched structure with a 'Y' at the end. Colonies on the surface of TPY agar reach a diameter of 1.0–2.5 mm after 2 days of incubation and are white, opaque, smooth and circular with entire edges; any embedded colonies show lens or elliptical shapes. Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and are able to survive under aerophilic and microaerophilic conditions. The temperature for growth is 25–40 °C; no growth at 20 or 44 °C. Grows at pH 5.5–7.5. The optimal growth conditions are pH 6.5 and 35 °C. Acid is produced from glycerol, D-glucose, D-fructose, D-mannose, salicin, cellobiose, maltose, lactose, sucrose, trehalose, raffinose and, to a lesser degree, from D-mannitol. Acid is also produced from L-arabinose, D-xylose, D-galactose, D-mannose, L-rhamnose, 2-ketogluconate, methyl α-D-glucopyranoside and, to a lesser degree, from *N*-acetylglucosamine and melezitose. Hydrolysis of aesculin and production of urease are positive. No fermentation involving erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, amygdalin, arbutin, melibiose, inulin, starch, glycogen, xylitol, gentiobiose, turanose, D-tagatose, D-fucose, D- or L-arabitol, gluconate or 5-ketogluconate. Enzymic activity is found for arginine dihydrolase, proline arylamidase, leucyl-glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase and histidine arylamidase. Esterase lipase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{14:0}$. The peptidoglycan type is L-Glu–L-Ala–L-Lys.

The type strain, MRM_5.18^T (=DSM 100201^T=JCM 30798^T), and the reference strain MRM_9.02 (=DSM 100342=JCM 30803) were isolated from the faeces of common marmosets. The DNA G+C content of the type strain is 63.7 mol%.

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PAPER 5

Michelini, S., Modesto, Pisi, A., Filippini, G., M., Biavati, B. & Mattarelli, P. (2015). *Bifidobacterium eulemuris* sp. nov., from the faeces of the black lemur (*Eulemur macaco*). *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijsem.0.000924.

Bifidobacterium eulemuris sp. nov., isolated from faeces of black lemurs (*Eulemur macaco*)

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Forty-three strains of bifidobacteria were isolated from the faeces of two adult black lemurs, *Eulemur macaco*. Thirty-four were identified as *Bifidobacterium lemurum*, recently described in *Lemur catta*. The nine remaining isolates were Gram-positive-staining, non-spore-forming, fructose-6-phosphate phosphoketolase-positive, microaerophilic, irregular rod-shaped bacteria that often presented Y- or V-shaped cells. Typing techniques revealed that these isolates were nearly identical, and strain LMM_E3^T was chosen as a representative and characterized further. Phylogenetic analysis based on 16S rRNA gene sequences clustered this isolate inside the genus *Bifidobacterium* and showed the highest levels of sequence similarity with *B. lemurum* DSM 28807^T (99.3 %), with *Bifidobacterium pullorum* LMG 21816^T and *Bifidobacterium longum* subsp. *infantis* ATCC 15697^T (96.4 and 96.3 %, respectively) as the next most similar strains. The *hsp60* gene sequence of strain LMM_E3^T showed the highest similarity to that of *Bifidobacterium stellenboschense* DSM 23968^T (93.3 %), and 91.0 % similarity to that of the type strain of *B. lemurum*. DNA–DNA reassociation with the closest neighbour *B. lemurum* DSM 28807^T was found to be 65.4 %. The DNA G+C content was 62.3 mol%. Strain LMM_E3^T showed a peptidoglycan structure that has not been detected in bifidobacteria so far: A3α L-Lys–L-Ser–L-Thr–L-Ala. Based on the phylogenetic, genotypic and phenotypic data, strain LMM_E3^T represents a novel species within the genus *Bifidobacterium*, for which the name *Bifidobacterium eulemuris* sp. nov. is proposed; the type strain is LMM_E3^T (=DSM 100216^T=JCM 30801^T).

Bifidobacteria represent one of the most important bacterial groups within the *Actinobacteria*, usually present in the gastrointestinal tract of humans and other mammals and the hindgut of honeybees (Biavati & Mattarelli, 2012) and bumblebees (Killer *et al.*, 2009, 2011). They have also been isolated from waste and dairy products, where the sources could have been faecal contamination and intentional probiotic addition, respectively (Mattarelli & Biavati, 2014). As lactic acid bacteria, bifidobacteria are considered probiotic strains because of their beneficial effects and their role in maintaining the health of their host (Turroni *et al.*, 2011).

As has been well documented, bifidobacteria are generally host-species-specific bacteria; indeed, their occurrence and species composition in different animals is quite variable, suggesting a separation into 'human' and 'animal' groups (Ventura *et al.*, 2004).

A work characterizing the faecal microbiome from non-human wild primates highlighted that primate microbiomes are host-specific and that differences observed among primate species of different families could not be accounted for solely by differences in habitat, but revealed an evolutionary dimension (Yildirim *et al.*, 2010). In recent years, compelling evidence has been obtained that, in addition to diet, primate gastrointestinal microbiomes are functionally linked to their vertebrate host taxa, and are perhaps species-specific or population-specific

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Abbreviation: F6PPK, fructose-6-phosphate phosphoketolase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial *hsp60*, *rpoB*, *dnaJ*, *clpC*, *rpoC* and *dnaG* gene sequences of strain LMM_E3^T are KP979748 and KP979742–KP979747, respectively.

Four supplementary figures are available with the online Supplementary Material.

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(Yildirim *et al.*, 2010). To date, the diversity of cultivable bifidobacteria in non-human primates is not fully understood.

Since 2012, several studies have focused on bifidobacteria isolated from non-human primates. As a result, 11 novel bifidobacterial species have been described from the prosimian *Lemur catta* (Modesto *et al.*, 2015) and from two New World monkey species, *Callithrix jacchus* and *Saguinus midas* (Endo *et al.*, 2012; Michelini *et al.*, 2015, 2016; Modesto *et al.*, 2014). From apes, the novel species *Bifidobacterium moukalabense* was isolated from the faeces of a wild gorilla (*Gorilla gorilla gorilla*) (Tsuchida *et al.*, 2014). Furthermore, *Bifidobacterium angulatum* was isolated from an adult female chimpanzee (*Pan troglodytes verus*) (Tsuchida *et al.*, 2014; Ushida *et al.*, 2010) and *Bifidobacterium dentium* and *Bifidobacterium adolescentis* were isolated from chimpanzee (*Pan troglodytes verus*) and orangutan (*Pongo pygmaeus*), respectively (D'Aimmo *et al.*, 2012); these three latter species are usually found in humans.

During the present study on cultivable bifidobacteria in members of the Lemuridae, 40 strains were isolated from fresh faecal samples of two adult black lemurs (*Eulemur macaco*) housed under semi-natural conditions in Parco Natura Viva, Verona, northern Italy.

The black lemur belongs to the family Lemuridae and occurs in almost all forested areas of Madagascar except for the spiny forests. Lemurs of the genus *Eulemur*, including *Eulemur macaco*, are usually considered to be generalized, opportunistic frugivore–folivores with a high degree of ecological flexibility and a wide dietary regime, which includes fruit, leaves, leaf stems, flowers, flower stems, spiders, spider webs, caterpillars, cicadas, insect cocoons and sometimes birds (Jolly *et al.*, 2006; Sauther *et al.*, 1999). They are monkeys with a gastrointestinal tract designed for variable yet moderate consumption of fibrous feeds and characterized by a simple stomach, a small intestine of moderate length and a caecum and colon of varied length, therefore allowing for a moderate fibre-fermentation capacity by microbes residing in the hindgut (Junge *et al.*, 2009).

Fresh faeces were collected from the ground using a sterile spoon, put into a sterile plastic tube and stored under anaerobic conditions in an anaerobic jar (Merck) at 4 °C. Samples were collected by the animal-care staff (keepers) during their routine cleaning of the enclosure, and were taken promptly to the laboratory (within 2 h). Samples of material (approx. 1–2 g) were serially diluted (10-fold) with peptone water (Merck) supplemented with cysteine hydrochloride (0.5 g l^{-1}); aliquots of each dilution (from 1 : 10 to 1 : 10^9) were inoculated onto TOS agar (Sigma Aldrich). After incubation, morphologically different colonies were picked randomly and restreaked for several generations to assure the purity of strains. Pure cultures were grown overnight in TPY broth under anaerobic conditions and suspended in a 10 % (w/v) sterile skimmed milk solution, supplemented with lactose (3 %) and yeast extract (0.3 %), and kept both freeze-dried and frozen at −120 °C.

Thirty-four isolates displayed cells with the peculiar C shape observed in *Bifidobacterium lemurum* DSM 28807^T. The other nine isolates showed rod-shaped cells, frequently forming filaments, with irregular contractions along the cells and bifurcations. All strains were cultivated under anaerobic conditions and maintained in TPY broth, pH 6.9, at 37 °C, unless indicated otherwise. Chromosomal DNA from all the isolated bacteria was obtained according to the method described by Rossi *et al.* (2000).

For discrimination of the isolates, molecular typing was carried out using the enterobacterial repetitive intergenic consensus sequences (ERIC) PCR, with the primer pair ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Ventura *et al.*, 2003), and the BOX-PCR with primer BOXAR1 (5′-CTACGGCAAGGCGACGCTGACG-3′) (Masco *et al.*, 2003). The ERIC-PCR mixture (20 µl) contained 3.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStartTaq plus DNA polymerase MasterMix kit; Qiagen), 30 ng DNA template and 2 µM each primer. Amplifications were performed in an Applied Biosystems Veriti thermal cycler and the temperature profile consisted of an initial denaturation step at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, at 48 °C for 30 s, and at 72 °C for 4 min; and a final extension step at 72 °C for 6 min. BOX-PCR was performed in a 20 µl amplification mixture containing 1.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStartTaq plus DNA polymerase MasterMix kit, Qiagen), an additional 0.05 mM of each dNTP, 70 ng DNA template and 2 µM each primer. The PCR amplification was performed in an Applied Biosystems Veriti Thermal cycler with the following temperature profile: an initial denaturation step at 94 °C for 3 min, 30 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 65 °C for 8 min, and a final extension step at 65 °C for 16 min. Aliquots of each amplification reaction (15 µl each) were separated by electrophoresis in 2 % (w/v) agarose gels at 7 V cm^{-1}. Gels were stained with ethidium bromide (0.5 µg ml^{-1}) and photographed under 260 nm UV light.

Two main groupings were identified on the basis of both ERIC-PCR and BOX-PCR banding profiles: group A, consisting of 34 isolates that displayed the same rep-PCR fingerprints as *B. lemurum* DSM 28807^T, and group B, consisting of nine strains with a homogeneous pattern different from that of group A (Fig. S1, available in the online Supplementary Material). The 16S rRNA gene from strain LMM_I1, selected as a representative strain for group A, was amplified and submitted to GenBank (accession no. KU171117); sequence similarity confirmed that group A belongs to *B. lemurum*. Strain LMM_E3^T was selected as a representative of group B and characterized further. In this study, morphological, biochemical and molecular characterizations of this isolate were carried out.

Using the primers listed in Table 1, the partial 16S rRNA, *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *clpC* and *rpoC* gene sequences of

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Table 1. Primers used for housekeeping gene amplifications

Primer	Sequence (5′–3′)
16S rRNA gene	
Bif285	GAGGGTTCGATTCTGGCTCAG
Bif261	AAGGAGGTGATCCAGCCGCA
hsp60	
HspF3	ATCGCCAAGGAGATCGAGCT
HspR4	AAGGTGCCGCGGATCTTGTT
rpoB	
BifF	TCGATCGGGCACATACGG
BifR2	CGACCACTTCGGCAACCG
dnaG	
DnaG-uni	CTGTGCCCGTTCCACGAC
DnaG-rev	CTCGATGCGCAGGTCGCA
dnaJ	
DnaJ1-uni	GAGAAGTTCAAGGACATCTC
DnaJ1-rev	GCTTGCCCTTGCCGG
clpC	
ClpC-uni	GAGTACCGCAAGTACATCGAG
ClpC-rev	CATCCTCATCGTCGAACAGGAAC
rpoC	
RpoC-uni	GTGCACTCGGTCCACAG
RpoC-rev	CATGCTCAACAACGAGAAG

strain LMM_E3^T were amplified by PCR. All reactions were performed in 20 µl PCR mixtures containing 1.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStartTaq plus DNA polymerase MasterMix kit; Qiagen), 0.1 µM each primer and 30 or 200 ng DNA template for the 16S rRNA gene and for each housekeeping gene, respectively. Amplifications were performed using a TGradient thermal cycler (Biometra). The 16S rRNA gene and all housekeeping genes were amplified with the same touchdown PCR, performed as follows: initial denaturation (95 °C, 5 min) for HotStartTaq plus activation; four cycles of denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s and extension at 72 °C for 90 s; 21 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 90 s; and 15 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s; the PCR was completed with a single elongation step (10 min at 72 °C). The resulting amplicons were separated on 2 % agarose gels, followed by ethidium bromide staining, and PCR fragments were purified using the NucleoSpinGel and PCR clean up kit (Macherey-Nagel) following the manufacturer's instructions. Amplicons representing partial 16S rRNA, *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *clpC* and *rpoC* genes were directly sequenced by Eurofins MWG Operon.

Almost-complete 16S rRNA gene sequence assembly was performed using CLC Sequence Viewer version 7.5 for MacOS. Sequences of closely related species were retrieved from the EMBL and GenBank nucleotide databases by comparison with database entries. Pairwise nucleotide sequence similarities were calculated with MatGat version 2.0 (Campanella *et al.*, 2003), using the Myers and Miller global alignment algorithm (Myers & Miller, 1988).

A phylogenetic tree based on a total of 61 partial 16S rRNA gene sequences including those of members of the genus *Bifidobacterium* and of related genera was reconstructed after sequence alignment with CLUSTAL Omega in CLC Sequence Viewer. The maximum-likelihood tree (Cavalli-Sforza & Edwards, 1967) and evolutionary distances were computed with Kimura's two-parameter method (Kimura, 1980) in MEGA 6.0 (Tamura *et al.*, 2013). The tree was rooted using *Micrococcus luteus* DSM 20030^T. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985) (Fig. S2).

Strain LMM_E3^T showed the highest 16S rRNA gene sequence similarity to *B. lemurum* DSM 28807^T (99.3 %), isolated by Modesto *et al.* (2015) from the ring-tailed lemur (*Lemur catta*), and to *Bifidobacterium pullorum* LMG 21816^T (96.4 %).

As the 16S rRNA gene sequence similarity of strain LMM_E3^T to known bifidobacterial species was higher than the recommended value (97 %) for species differentiation (Kim *et al.*, 2014), DNA–DNA hybridization between strain LMM_E3^T and *B. lemurum* DSM 28807^T was carried out. Estimation of the level of relatedness between *B. lemurum* DSM 28807^T and strain LMM_E3^T was determined by the DSMZ, Braunschweig, Germany. Using a Constant Systems TS 0.75 kW (IUL Instruments), cells were disrupted and DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was performed according to De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). Strain LMM_E3^T shared 65.4 and 67.2 % (direct and reciprocal values, respectively) DNA–DNA relatedness with *B. lemurum* DSM 28807^T; these values support the conclusion that the strains do not belong to the same species when the recommendation of a threshold value of 70 % DNA–DNA relatedness for the definition of bacterial species is considered (Wayne *et al.*, 1987). However, these values suggest that the strains are closely related.

The phylogenetic location of the novel strain LMM_E3^T was also verified by the analysis of six genetic markers, *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *clpC* and *rpoC*, that have proven to be discriminative for classification of the genus *Bifidobacterium* (Jian *et al.*, 2001; Kim *et al.*, 2010; Ventura *et al.*, 2006). Indeed, multilocus sequence analysis (MLSA) is a reliable and robust technique for the identification and classification of bacterial isolates to the species level, representing an alternative to 16S rRNA gene sequence analysis (Martens *et al.*, 2008).

The partial *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *clpC* and *rpoC* gene sequences of strain LMM_E3^T and of its closest relatives

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retrieved from the DDBJ/GenBank/EMBL databases were aligned by using the MAFFT program, at CBRC (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). The Gblocks program (version 0.91b), a server tool at the Castresana laboratory (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was then used to eliminate poorly aligned positions and divergent regions of DNA alignments, facilitating the phylogenetic analysis (Talavera & Castresana, 2007).

Approximately 594 nt of the partial *hsp60* gene sequences of strain LMM_E3^T and related species (retrieved from the EMBL and GenBank nucleotide databases) were used to reconstruct a phylogenetic tree by using the neighbour-joining method, with Kimura's two-parameter method as a substitution model. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Fig. 1). The *hsp60* gene sequence of strain LMM_E3^T showed the highest similarity to those of *Bifidobacterium stellenboschense* DSM 23968^T and *Bifidobacterium scardovii* LMG 21589^T (93.3 and 92.2 %, respectively), but only 91.0 % similarity to that of *B. lemurum* DSM 28807^T.

The highest levels of similarity for all partial housekeeping gene sequences of strain LMM_E3^T and its closest relatives were: for *hsp60*, 93.3 % with *B. stellenboschense* JCM 17298^T, 92.2 % with *B. scardovii* JCM 12489^T and 91.0 % with *B. lemurum* DSM 28807^T; for *rpoB*, 97.7 % with *B. lemurum* DSM 28807^T and 91.7 % with *Bifidobacterium aesculapii* DSM 26737^T; for *clpC*, 97.8 % with *B. lemurum* DSM 28807^T and 88.0 % with *Bifidobacterium longum* subsp. *infantis* ATCC 15697^T; for *dnaJ*, 92.5 % with *B. lemurum* DSM 28807^T and 79.8 % with *Bifidobacterium adolescentis* 22L (Duranti *et al.*, 2014); for *dnaG*, 95.1 % with *B. lemurum* DSM 28807^T and 83.8 % with *B. adolescentis* 22L; and for *rpoC*, 96.9 % with *B. lemurum* DSM 28807^T, 88.8 % with *Bifidobacterium animalis* subsp. *lactis* ATCC 27673 and 88.1 % with *B. adolescentis* 22L.

To conduct MLSA, the six housekeeping-gene sequences were concatenated, yielding approximately 4188 positions. The maximum-likelihood method, with Kimura's two-parameter method as a substitution model, was used to reconstruct a phylogenetic tree based on the concatenated sequences in MEGA 6. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Fig. S3).

Phylogenetic tree reconstruction was also performed on the concatenated deduced amino acid sequences of the housekeeping genes by bootstrap analysis of 1000 replicates with the maximum-likelihood method. Evolutionary distances were computed using Kimura's two-parameter method and are in the units of the number of base substitutions per site (Fig. 2). In this tree, LMM_E3^T is paraphyletic with respect to the monophyletic *B. adolescentis* and *B. longum* groups, in which *B. lemurum* DSM 28807^T is included.

hsp60 RFLP-PCR analysis, developed by Baffoni *et al.* (2013), was described as a rapid tool for the efficient identification of bifidobacteria at the species and subspecies levels. The method was used to discriminate LMM_E3^T from *B. lemurum* DSM 28807^T after virtual digestion of the obtained *hsp60* gene sequences with *Hae*III according to Baffoni *et al.* (2013). The theoretical fragment patterns were different, 32–42–60–104–139–158 bp for LMM_E3^T and 10–42–81–104–139–158 bp for *B. lemurum* DSM 28807^T, suggesting the placement of the two strains in separate taxa (Fig. S4).

DNA G+C content estimation from bacterial chromosomal DNA of strain LMM_E3^T was performed by the DSMZ. Briefly, DNA was purified on hydroxyapatite according to the procedure of Cashion *et al.* (1977) and enzymically hydrolysed by the method of Mesbah *et al.* (1989). The resulting deoxyribonucleosides were analysed by HPLC (Tamaoka & Komagata, 1984). Strain LMM_E3^T showed a DNA G+C content of 62.3 mol%, within the range of values reported for the genus *Bifidobacterium* (52–67 mol%; Biavati & Mattarelli, 2012; Killer *et al.*, 2010).

Morphological, cultural and biochemical characterization of the isolate was performed anaerobically at 37 °C according to standard techniques unless otherwise stated.

The different morphologies of cells of strain LMM_E3^T and its closest relative *B. lemurum* DSM 28807^T, as revealed by phase-contrast microscopy, are shown in Fig. 3(a, b): LMM_E3^T showed rod-shaped cells, while *B. lemurum* DSM 28807^T showed small, peculiar, C-shaped cells. The morphological characteristics of LMM_E3^T, as determined by scanning electron microscopy (SEM), are shown in Fig. 3(c, d). For SEM observations, strains were cultured in TPY broth at 37 °C for 48 h under anaerobic conditions. After culturing, pelleted cells were spotted on Whatman no. 1 qualitative filter paper and air-dried. Samples were mounted on aluminium stubs with silver glue and coated with gold–palladium film using an ion-sputtering unit (Emitech K500); observations were made in a Philips 515 SEM at 7–10.0 kV.

The range of temperature for growth was determined by measuring the OD_{600} of cultures incubated at 20, 25, 30, 35, 37, 40, 42, 45 and 47 °C for 48 h under anaerobic conditions. The sensitivity of the strain to low pH was assessed in TPY broth at 37 °C under anaerobic conditions for 48 h at pH 3.5, 4.0, 4.5, 5.0 and 5.5. Sensitivity to oxygen was tested under aerobic and microaerophilic conditions (CampyGen; Oxoid) using both TPY agar and TPY soft agar (0.6 %).

Haemolytic activity was determined on Columbia blood agar (Biolife) at 37 °C under anaerobic conditions for 48 h (Pineiro & Stanton, 2007). Spore staining was performed using malachite green dye. Phase-contrast microscopy (Zeiss) was used to observe the morphology of individual cells, as well as spore staining.

Gram staining and catalase and oxidase activities were determined with cells grown on TPY agar at 37 °C for 48 h under anaerobic conditions using individual Gram

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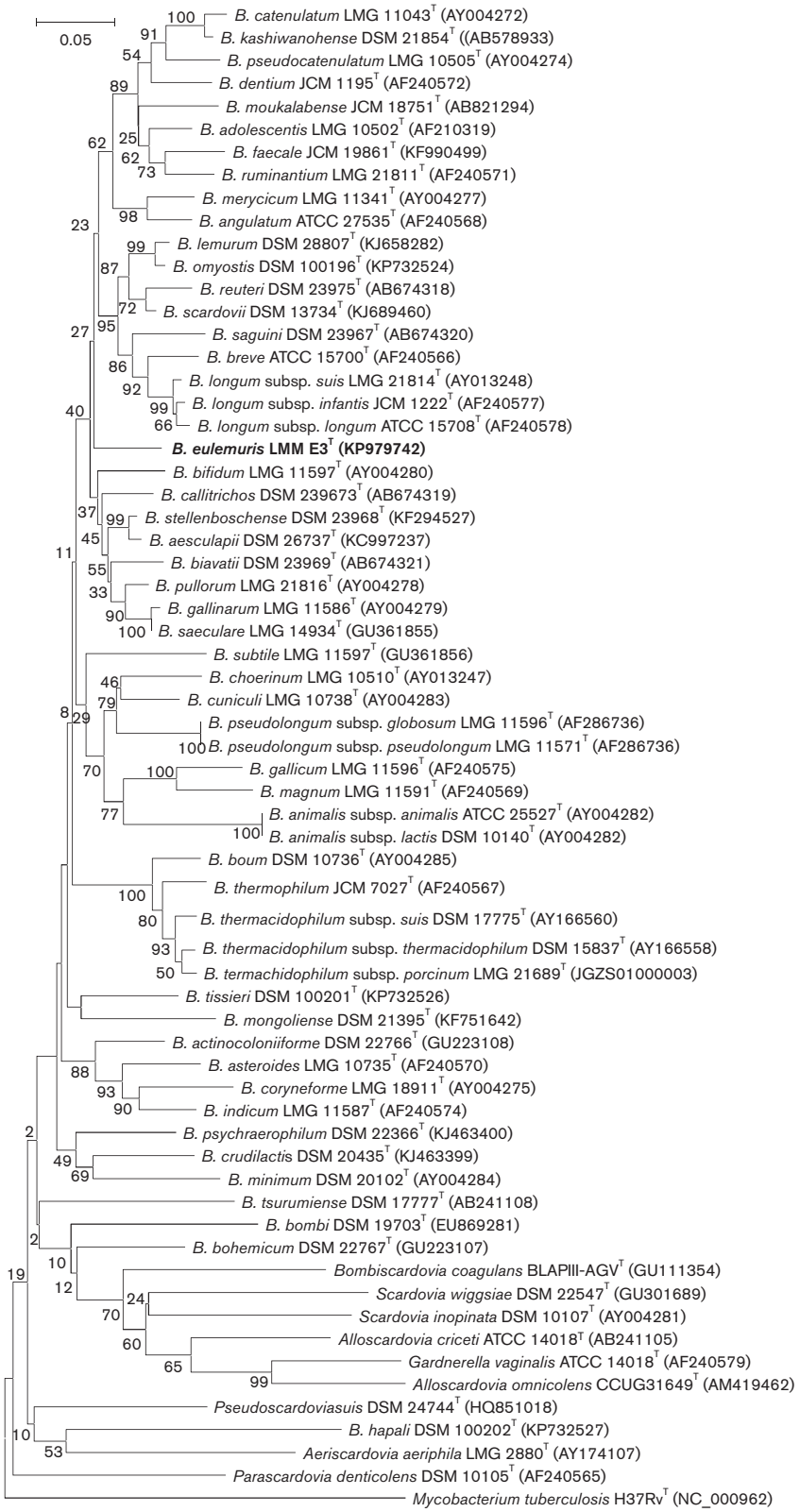

Fig. 1. Phylogenetic tree based on *hsp60* gene sequences showing the relationship of the novel strain with strains of closely related species. The tree was reconstructed by the neighbour-joining method on the basis of a comparison of approximately 559 positions, and the sequence of *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; values above 50 % are shown. Bar, 0.05 substitutions per nucleotide position.

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Fig. 2. Phylogenetic tree based on concatenated deduced amino acid sequences of the *hsp60*, *clpC*, *dnaG*, *dnaJ*, *rpoB* and *rpoC* genes, showing the relationship of the novel strain LMM_E3^T with strains of closely related species. The tree was reconstructed by the maximum-likelihood method and the sequences of *Mycobacterium tuberculosis* H37Rv^T were used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; values above 50 % are shown. Bar, 0.01 substitutions per nucleotide position.

staining reagents (Merck Millipore), a 3 % (v/v) hydrogen peroxide solution and cotton swabs impregnated with *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine dihydrochloride and dried (Oxibioswab; Biolife), respectively. The motility of the strain was determined by stabbing the culture into TPY medium containing 0.4 % agar, knowing that motile strains show a diffuse growth spreading from the line of inoculation. Fermentation products (short-chain fatty acids) were analysed according to the method described by Holdeman *et al.* (1977). Briefly, after growth in TPY broth with 1 % glucose, volatile acids were extracted with diethyl ether. A Carlo Erba 5300 gas chromatograph, with a Nukol capillary column (30 cm) at 170 °C, flame-ionization detector and hydrogen carrier gas, was used for the analysis. All strains tested fermented glucose to acetate and lactate in a ratio of 1.5 : 1.

Substrate utilization and enzyme production by LMM_E3^T and its nearest relatives *B. lemurum* DSM 28807^T, *B. stellenboschense* DSM 23968^T and *B. scardovii* LMG 21589^T were tested with API 50 CH and Rapid ID 32 test

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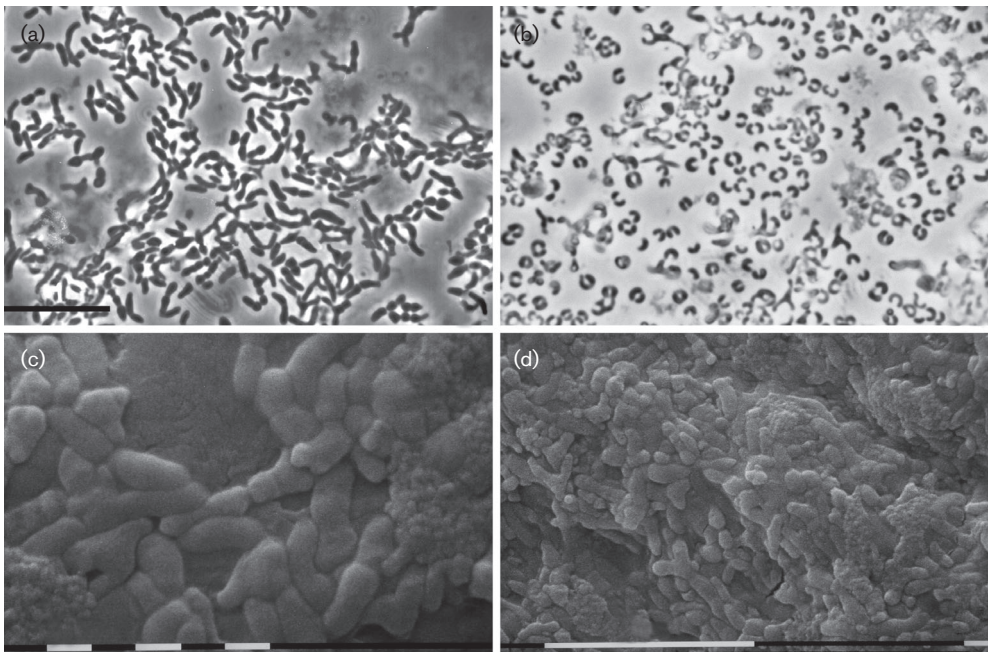

Fig. 3. (a, b) Phase-contrast photomicrographs showing the cellular morphology of strain LMM_E3^T (a) and *B. lemurum* DSM 28807^T (b). (c, d) Scanning electron photomicrographs of strain LMM_E3^T. Bars, 10 μm (a, d) and 1 μm (c).

kits (bioMérieux). The results are summarized in Table 2. The relevant differences between LMM_E3^T and the closest relative *B. lemurum* DSM 28807^T are as follows. Strain LMM_E3^T produced acid from D-sorbitol, whereas *B. lemurum* DSM 28807^T produced acid from cellobiose, melezitose and glycogen. Moreover, arginine dihydrolase, alkaline phosphatase, pyroglutamic acid arylamidase and glutamyl glutamic acid arylamidase activities were negative for LMM_E3^T but positive for *B. lemurum* DSM 28807^T (Table 2).

Bifidobacteria and members of related genera possess a peculiar enzyme, fructose-6-phosphate phosphoketolase (F6PPK), which degrades hexoses via the F6PPK pathway and is considered a taxonomic marker for identification of species of *Bifidobacterium* and related genera (Biavati & Mattarelli, 2012). Detection of F6PPK activity was carried out according to the method described by Scardovi (1986) and modified by Orban & Patterson (2000). All tested isolates possessed F6PPK activity.

Following the protocol of Schumann (2011), the cell-wall murein composition of strain LMM_E3^T was examined by the DSMZ. The total hydrolysate of the peptidoglycan revealed the presence of muramic acid and the amino acids lysine, alanine, glutamic acid, serine and threonine. In addition, the peptides L-Ala–D-Glu, L-Lys–D-Ala, L-Lys–L-Ser, D-Ala–L-Lys–L-Ser, L-Ser–L-Thr and L-Ala–D-Ala were detected. After hydrolysis under stronger conditions (100 °C, 4 M HCl, 16 h), the peptides were almost completely hydrolysed and quantitative analysis resulted in the following ratio: 1.4 D-Ala; 1.2 L-Ala; 1.4 L-Thr; 0.8 L-Ser; 1.0 D-Glu; 2.8 L-Glu; 2.0 L-Lys. From these data, it was concluded that strain LMM_E3^T displayed the following peptidoglycan type: A3α L-Lys–L-Ser–L-Thr–L-Ala. This proposed peptidoglycan structure is different from that of *B. lemurum*; it is typical of several species of *Arthrobacter* but has not yet been detected in bifidobacterial species to the best of our knowledge. This is a strong point in favour of the assignment of LMM_E3^T to a novel species, since it is in agreement with the most recent guidelines described in the minimal standards for description of new taxa of the genera *Bifidobacterium* and *Lactobacillus* and related genera (Mattarelli *et al.*, 2014).

Based on phylogenetic analyses of concatenated deduced amino acid sequences of *hsp60*, *rpoB*, *dnaG*, *dnaJ*, *rpoC* and *clpC*, DNA–DNA hybridization analysis and other data, such as *hsp60* RFLP-PCR, DNA G+C content and peptidoglycan structure, strain LMM_E3^T is genetically and phenotypically discernible from currently recognized species of bifidobacteria and thus represents a novel species, for which we suggest the name *Bifidobacterium eulemuris* sp. nov.

Description of *Bifidobacterium eulemuris* sp. nov.

Bifidobacterium eulemuris (eu.le.mu′ris. N.L. gen. n. *eulemuris* of *Eulemur macaco*, referring to the primate host of the type strain).

Cells grown in TPY broth are rod-shaped, frequently forming filaments, with irregular contractions along the cells

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Table 2. Differential characteristics between novel strain LMM_E3^T and its closest phylogenetic relatives

Strains: 1, LMM_E3^T; 2, *B. lemurum* DSM 28807^T; 3, *B. stellenboschense* DSM 23968^T; 4, *B. scardovii* LMG 21589^T. +, Positive; w, weak; −, negative. All data were obtained in this study.

Characteristic	1	2	3	4
Fermentation of:				
Glycerol	w	−	w	w
D-Arabinose	−	−	w	+
L-Arabinose	w	+	+	+
D-Xylose	+	+	+	−
D-Adonitol	w	−	−	−
Methyl β-D-xylopyranoside	+	−	−	−
D-Galactose	−	+	+	+
D-Fructose	w	w	w	+
D-Mannitol	+	w	+	−
D-Sorbitol	+	−	+	−
Methyl α-D-glucopyranoside	+	w	w	+
N-Acetylglucosamine	w	−	−	−
Amygdalin	+	w	w	+
Cellobiose	−	+	−	+
Lactose (bovine origin)	w	+	w	+
Trehalose	w	w	−	+
Melezitose	−	+	+	+
Glycogen	−	+	w	−
Turanose	+	w	+	+
Potassium gluconate	−	−	−	w
Potassium 2-ketogluconate	w	+	−	−
Potassium 5-ketogluconate	+	+	−	−
Enzymic activity				
Urease	−	−	−	w
Arginine dihydrolase	−	+	−	−
α-Glucosidase	+	w	w	+
α-Arabinosidase	−	−	−	+
β-Glucuronidase	−	−	−	w
N-Acetyl-β-glucosaminidase	−	−	−	w
α-Fucosidase	−	−	−	+
Alkaline phosphatase	−	+	+	−
Proline arylamidase	+	+	+	−
Leucyl glycine arylamidase	w	+	+	−
Pyroglutamic acid arylamidase	−	+	+	+
Tyrosine arylamidase	+	w		+
Alanine arylamidase	+	w		w
Glutamyl glutamic acid arylamidase	−	+		−

and bifurcations (2–5 μm). They are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and microaerophilic. Spatially well separated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.5 mm in diameter after 3 days of incubation. The temperature range is 35–42 °C; no growth occurs at 30 or 47 °C. The optimum temperature for growth is 37–42 °C. Grows at pH 5.5–7.0, with an optimum at pH 6.5–7.0. Can grow in milk, under both microaerophilic and anaerobic conditions. Acid is produced from D-glucose, D-ribose, D-xylose, methyl β-D-xylopyranoside, D-mannose, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, melibiose, raffinose, gentiobiose, turanose and potassium 5-ketogluconate. Acid may be produced from glycerol, L-arabinose, D-adonitol, D-fructose, *N*-acetylglucosamine, lactose, trehalose and potassium 2-ketogluconate. Acid is not produced from D-arabinose, L-xylose, D-galactose, L-sorbose, methyl α-D-mannopyranoside, cellobiose, melezitose, glycogen or potassium gluconate. Results from Rapid 32ID tests reveal enzymic activity of α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, leucine arylamidase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Leucyl glycine arylamidase is produced weakly. Negative for enzymic activities of urease, arginine dihydrolase, α-arabinosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, alkaline phosphatase, pyroglutamic acid arylamidase and glutamyl glutamic acid arylamidase, reduction of nitrates and production of indole. Aesculin is hydrolysed. The peptidoglycan type is A3α L-Lys–L-Ser–L-Thr–L-Ala. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. longum* subgroup of the genus *Bifidobacterium*.

The type strain, LMM_E3^T (=DSM 100216^T=JCM 30801^T), was isolated in February 2014 from a fresh faecal sample of an adult black lemur (*Eulemur macaco*) that was housed under semi-natural conditions in the Parco Natura Viva, Verona, northern Italy. The DNA G+C content of the type strain is 62.3 mol%.

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DRAFT 1

Michelini, S., Modesto, Pisi, A., Filippini, G., M., Biavati, B. & Mattarelli, P. (2015). *Bifidobacterium aeriphilum* sp. nov. *Bifidobacterium avesanii* sp. nov. and *Bifidobacterium ramosus* sp. nov.: three novel taxa from the faeces of cotton-top tamarin (*Saguinus oedipus* L.). (Submitted)

Bifidobacterium aeriphilum sp. nov. *Bifidobacterium avesanii* sp. nov. and *Bifidobacterium ramosum* sp. nov.: three novel taxa from the faeces of cotton-top tamarin (*Saguinus oedipus* L.)

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Running title: *Bifidobacterium aeriphilum, Bifidobacterium avesanii* and *Bifidobacterium ramosum* sp. nov. in the faeces of the cotton-top tamarin.

Journal's contents category: New taxa –*Actinobacteria*

Key words: New species, *Bifidobacterium, Bifidobacterium aeriphilum, Bifidobacterium avesanii, Bifidobacterium ramosum,* cotton-top tamarin, *Saguinus oedipus.*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp60*, *rpoB*, c*lpC, dnaJ* and *dnaG* partial gene sequences of *B. aeriphilum* TRE_17^T and TRE_26 are KU051444, KU051445 (16S), KU194202 (hsp60), KU058184 and KU058185rpoB, KU171118 and KU171119 (ClpC), KU179316 and KU179317 (dnaJ), KU171122 and KU171123 (dnaG); for *B. avesanii* TRE_C^T are KU051446 (16S), KU194203 (hsp60), KU058186 (rpoB), KU171120 (ClpC), KU179318 (dnaJ), KU171124 (dnaG), ; for *B. ramosum* TRE_M^T are KU051447 (16S), KU 194204 (hsp60) KU058187 (rpoB), KU171121 (ClpC), KU179319 (dnaJ) KU171125 (dnaG),.

ABSTRACT

Forty-five microorganisms were isolated in a selective medium for bifidobacteria from one faecal sample of an adult subject of the cotton-top tamarin (Saguinus Oedipus L.), a New World monkey. All isolates resulted gram-positive-staining, catalase-negative, facultative anaerobic, fructose-6-phosphate phosphoketolase (F6PPK) positive and asporogenous rod-shaped bacteria. In this study only eight out of forty-five strains were deeply characterized whereas the other ones are currently under investigation. They were grouped by BOX-PCR in 3 clusters: Cluster I (TRE_17^T, TRE_7, TRE_26, TRE_32, TRE_33, TRE_I); Cluster II (TRE_C^T); Cluster III (TRE_M^T). Comparative analysis of 16S rRNA gene sequences confirmed results from cluster analysis and revealed relatively low levels of similarities to each other (mean value of 95%) and to members of the genus Bifidobacterium. All eight isolates showed the highest level of 16S rRNA gene sequence similarities with Bifidobacterium scardovii DSM 13734^T (mean value 96.6%). Multilocus Sequence Analysis (MLSA) of five housekeeping genes, (hsp60, rpoB, clpC, dnaJ and dnaG) supported their independent phylogenetic position to each other and to related species of Bifidobacterium. The GC contents were 63.2%, 65.9% and 63.0% for Cluster I, Cluster II and Cluster III, respectively. Petidoglycan types were A3α L-Lys – L-Thr – L-Ala, A4β L-Orn (Lys) – D-Ser – D-Glu and A3β L-Orn – L-Ser – L-Ala in Cluster I, II and III, respectively. Based on the data provided, each Cluster represents a novel taxon for which the names Bifidobacterium aeriphilum sp. nov. (type strain: TRE_17^T =DSM 100689 and =JCM 30941; and reference strain: TRE_26 =DSM 100690 and =JCM 30942), Bifidobacterium avesanii sp. nov. (type strain: TRE_C^T =DSM 100685 and =JCM 30943) and Bifidobacterium ramosum sp. nov. (type strain: TRE_M = DSM 100688 and =JCM 30944) are proposed.

INTRODUCTION

All mammals rely upon mutualistic gut microbial communities that play a role in the host diet, in the nutrient adsorption, mucosal defences, and immune system development, thus reflecting the coevolution of the microorganisms with their animal host and the diet of the host [2]. Therefore, the study of the intestinal bacterial diversity is a very important topic to better understand the relationship between bacterial communities and their hosts, and to determine the relationship between the microbial community structure and function.

Previous studies have proven that the microbiomes of non-human primates exhibit a much higher similarity with those of primates than with other animals [16]. Therefore, the study of the microbiota from these non-human primates provides important insights into the reflection of their features in humans. However, only a few reported culture-independent studies on faecal microbiota of non-human primates are available, and these have mainly focused on higher primates, such as the baboon, gorilla, [2] orangutan and chimpanzee [16,26,40]..

Interestingly, a recent study on gut microbiome assembly of three species (*Varecia variegata, Lemur catta,* and *Propithecus coquereli*) in the primate clade of *Lemuriformes*, [20] showed differences in the distributions of their dominant phyla respect to other non-human primates. Specifically, lemurs appear to harbour ratios of *Proteobacteria* and *Firmicutes* more similar to *Pan* species than to either Gorilla species [26] or to *Nycticebus pygmaeus* (pygmy loris, the only other prosimian whose gut microbiota has been studied to date [2]) while *Bacteroidetes* shows the opposite relationship. Lemur gut microbiomes contained two bacterial lineages associated with humans consuming a distinctly non-Western diet, probably due to the higher prevalence of plants and produce in non-Western diets, thus yielding the similarity to those species found in lemurs [20].

Several works have suggested the importance of isolating and identifying novel strains of the genus *Bifidobacterium* from various animals including humans to understand how they are mostly distributed [5,37] and even more which are their phenotypic and genotypic characteristics, thus allowing the reconstruction of a more robust bifidobacterial phylogeny.

Bifidobacteria represent one of the most important bacterial groups within the *Actinobacteria*. Ecological studies revealed their presence in the gut of a wide variety of animals (e.g. mammals, birds, ungulates, lagomorphs and rodents) and insect pollinators [1,11,12,15,29].To date, studies on bifidobacterial distribution in New World monkeys and lemurs have revealed the presence of twelve novel species belonging to this genus [5,22–25]. Bifidobacteria were also isolated from waste and dairy products, where the sources could have been faecal contamination and intentional probiotic addition, respectively [19]. Therefore, bifidobacteria are usually referred to as host- and animal-species-specific bacteria and are classified as 'human type' and 'non-human type' groups and others.[37,39].

During a study on diversity of cultivable bifidobacteria in *Callitrichidae*, one adult subject of the cotton-top tamarin (*Saguinus oedipus*), housed under semi-natural conditions in Parco Natura Viva (Verona, North Italy) had been considered. The cotton-top tamarin is a small New World monkey whose diet is principally composed of insects, ripe fruits, plant exudates, floral nectar, reptiles and amphibians [7,36].

In February 2014, individual fresh faeces were collected from the ground using a sterile spoon, put into a sterile plastic tube and stored under anaerobic conditions in an anaerobic jar (Merck) at 4 °C. Samples of fresh faeces were collected by the animal-care staff (keepers) during their routine cleaning of the enclosure, and were taken promptly to the laboratory (within 2 h). Samples of the material, of approx. 1–2 g, were serially diluted with peptone water (Merck) supplemented with cysteine hydrochloride (0.5 g l^{-1}), and aliquots of each dilution were inoculated onto TPY [31] supplemented with mupirocine (100mg/L) (Applichem).

After incubation, morphologically different colonies were randomly picked-up and re-streaked for several generation in order to isolate purified individual bacterial strains.

A total of forty-five isolates were obtained. All strains were subcultured on TPY broth and their cells were suspended in a 10% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), freeze-dried and kept frozen at −120 °C. For all experiments, the strains were cultivated under anaerobic conditions in anaerobic jars (Merck) and maintained in TPY broth, at pH 6.9 and 37 °C, unless indicated otherwise. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD).

Chromosomal DNA of the isolated strains was extracted with the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions with slight modifications. Briefly, pelletted cells from overnight cultures were washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), re-suspended in TE containing 50 mg lysozyme ml^{-1} and incubated in a water bath at 37°C for 80 minutes. After adding 600 µl of Nucleic Lysis Solution to the lysate, an incubation step of 15 minutes at 80°C was also carried out. All subsequent manipulations were performed according to manufacturer's instruction. The DNA concentration was determined spectrophotometrically from the A_{260} and the purity of each sample was estimated by determining the A_{260}/A_{280} ratio.

For discrimination of the isolates, BOX-PCR fingerprinting was carried out using the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Fig. S1) [18]. The 20 µl of final reaction mixture volume contained 1.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each dntps, deoxynucleoside triphosphate, (HotStartTaq plus DNA polymerase MasterMix kit, Qiagen), additional 0.05 mM of dNTPs, 70 ng of DNA template and 2 µM of primer. The PCR amplification was performed in an Applied Biosystem Verity Thermal cycler (Applied Biosystems, Foster City, CA) with the following temperature profile: initial denaturation step at 94°C for 3 min, 30 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min an extension at 65°C for 8 min, and a final extensions step at 65°C for 16 min. Amplicons (20 µl) were fractioned by electrophoresis on a 2% agarose (w/v) gel at a voltage of 7 V/cm. Gels were ethidium bromide stained (0.5 µn/ml) and the fingerprinting profiles visualized under 260 nm UV light (Molecular Imager Gel

Doc XR (BIO-Rad). The banding patterns from BOX-PCR were first acquired by Image lab software (BioRad) and subsequently analysed with Gel Compare II software version 6.6.11 (Applied-Maths, Ghent, Belgium). The similarities between strains were calculated using the Jaccard similarity index and cluster analysis was obtained by means of the Unweighted Pair Group Method with Arithmetic Average (UPMGA) clustering algorithm.
The eight isolates were separated in three Clusters (Fig. S1) viz. Cluster I (strains: TRE_7, TRE_17^T, TRE_26, TRE_32, TRE_33, TRE_I), Cluster II (strain TRE_C ^{T}) and Cluster III (strain TRE_M^T). Given that the isolates of Cluster 1 revealed similar BOX-PCR profiles (see Fig. S1), strains TRE_17^T and TRE_26 were selected as representatives and characterized further.

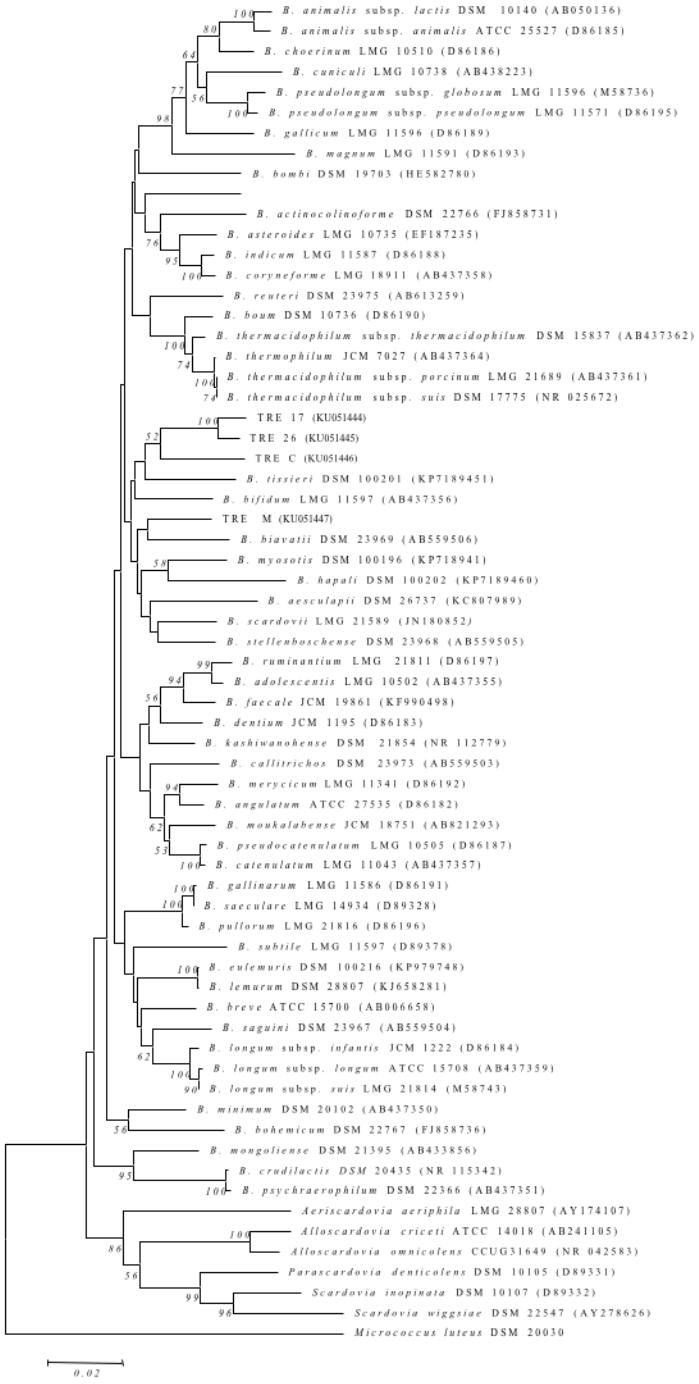
Morphological and biochemical profiles, growth parameters and molecular characterisation were carried out and evaluated for the selected strains.
The almost complete 16S rRNA gene sequence of strains were amplified using primer pair Bif285 (5'-GAGGGTTCGATTCTGGCTCAG-3') and Bif261 (5'-AAGGAGGTGATCCAGCCGCA-3') [13]. Partial *hsp60, rpoB, dnaJ, dnaG, clpC and rpoC* gene sequences were obtained using the primer pairs HspF3 (5'-ATCGCCAAGGAGATCGAGCT-3') and HspR4 (5'-AAGGTGCCGCGGATCTTGTT-3'), BifF (5'-TCGATCGGGCACATACGG-3') and BifR2 (5'-CGACCACTTCGGCAACCG-3') [13], DnaJ1-uni (5'-GAGAAGTTCAAGGACATCTC-3') and DnaJ1-rev (5'GCTTGCCCTTGCCGG-3'), ClpC-uni (5'-GAGTACCGCAAGTACATCGAG-3') and DnaG-uni (5'-CTGTGCCCGTTCCACGAC-3' and DnaG-rev (5'-CTCGATGCGCAGGTCGCA-3'), and ClpC rev (5'-CATCCTCATCGTCGAACAGGAAC-3') [38] respectively.

All the reactions were performed in 20 µl of PCR mixtures containing 1.5 mM of $MgCl_2$, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each deoxynucleoside triphosphate (HotStartTaq plus DNA polymerase MasterMix Kit; Qiagen), 0.1 µM of each primer and 30 ng or 200 ng of DNA template for 16S rRNA gene and for each housekeeping gene, respectively. Amplifications were performed in a TGradient thermal cycler (Biometra). Amplification of the 16S rRNA gene and of all housekeeping genes were obtained with a touch down PCR performed as follows: initial denaturation (95 °C, 5 min) for HotStart Taq plus activation; 4 cycles with denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s, and extension at 72 °C for 90 s; 21 cycles with denaturation at 94 °C for 60 s, annealing at 60°C for 60 s, and extension at 72 °C for 90 s; 15 cycles with denaturation at 94 °C for 60 s, annealing at 58°C for 60 s, and extension at 72 °C for 90 s; the PCR was completed with a single elongation step (10 min at 72 °C).
Resulting amplicons were separated on 2 % agarose gels, followed by ethidium bromide staining and PCR fragments were purified using the NucleoSpin Gel and PCR clean up kit (Macherey-Nagel, Duren, Germany) following manufacturer's instructions. The 16S rRNA, *hsp60, rpoB, dnaG, dnaJ,* and *clpC* gene partial sequences were amplified, purified and directly sequenced by Eurofins MWG Operon.

Almost-complete 16S rRNA gene sequence assembly was performed using CLC Sequence Viewer version 7.5 for Mac OS (CLC, Inc., Aarhus, Denmark). After editing, the closest known relatives of the novel strains were determined by comparison with database entries, and sequences of members of closely related species were retrieved from the EMBL and GenBank nucleotide databases. Pairwise nucleotide sequence similarity values were calculated using the lalign program (http://embnet.vital-it.ch/software/LALIGN_form.html), which provides a web-based tool.
The 16S rRNA gene sequences (about 1350 bp) of strains TRE_17^T and TRE_26, TRE_C ^{T}, TRE_M^T and of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned using Clustal Omega in CLC Sequence Viewer. A phylogenetic tree based on a total of 65 partial 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium* and of related genera, was reconstructed with the neighbour-joining method [30] and the evolutionary distances were computed by the Kimura 2-parameter method [14] using MEGA VERSION 6.0 [35]. The tree was rooted using *Micrococcus luteus* DSM 20030^T (Fig. 1). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates [6] and the

maximum-likelihood [4] method using MEGA VERSION 6.0 [35] was used to confirm the tree topology (Fig. S2).

Figure 1. Phylogenetic relationship between strains TRE_17^T, TRE_26, TRE_C^T and TRE_M^T and members of related *Bifidobacterium* species based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method and rooted with *Micrococcus luteus* DSM 20030^T. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap percentages above 50 are given at branching points



All strains of Cluster 1, 2 and 3 showed low 16S rRNA gene sequence similarities to each other and to known bifidobacteria and the highest values were found to *Bifidobacterium scardovii* DSM 13734^T: 95.8%, 95.8% and 95.9% respectively (Table 1).

Table 1. Pairwise sequences similarities of 16S rRNA, *hsp60, rpoB, ClpC, dnaG* and *dnaJ* partial genes of strains isolated from the cotton top tamarin and their closest relatives.

	16S rRNA				***hsp60***				***rpoB***				***ClpC***				***DnaG***				***DnaJ***			
	TRE17	TRE26	TREC	TREM	TRE17	TRE26	TREC	TREM	TRE17	TRE26	TREC	TREM	TRE17	TRE26	TREC	TREM	TRE17	TRE26	TREC	TREM	TRE17	TRE26	TREC	TREM
TRE_17		99.4	94.7	94.7			93.0	91.9		98.7	90.9	86.0		99.1	87.5	94.3						98.4		72.9
TRE_26	99.4		95.9	96.1					98.7		90.2	85.4	99.1		88.0	94.8	98.8		85.2		98.4			72.7
TRE_C	94.7	95.9		95.6	93.0			94.7	90.9	90.2		87.6	87.5	88.0		94.3	79.8	85.2						
TRE_M	94.7	96.1	95.6		91.9		94.7		86.0	86.0	87.6		94.3	94.8	94.3		82.5	86.2	86.4			72.7		
***B. scardovii* DSM 13734**	96	96.9	95.8	96.1				93.8	93.0	92.7			90.5	90.9	88.2	91.9			86.2	86.8	79.7	87.8		
***B. reuteri* DSM 23968**	95.1	96.5																						
***B. tissieri* DSM 100201**	94.6	95.5																						
***B. aesculapii* DSM 26737**					93.3		95.8	94.6	91.0	91.7	89.8								86.8	84.8				
***B. pullorum* DSM 20433**					93.1																			
***B. callithricos* DSM 28792**					92.2																			
***B. bifidum* DSM 20456**									90.5	89.9	89.9		87.6	88.1	87.8	86.8					79.9	79.6		
***B. biavatii* DSM 23969**																					86.5	87.2		
***B. kashiwanohense* DSM 21854**			95.6																					
***B.pseudocatenulatum* DSM 20438**			95.5																					
***B. longum* subsp. *infantis* DSM 20088**				95.5											86.9									83.6
***B. longum* subsp. *suis* DSM 20211**				95.4																				
***B. saeculare* DSM 6531**							95.3	94.9																
***B. gallinarum* DSM 20670**							95.3																	
B. pseudolongum PV 8-2											91.0													
***B. longum* subsp. *longum* DSM 20097**												92.2					79.5	83.0						
***B. angulatum* DSM 20098**												89.7												
***B. breve* DSM 16604**												91.5												82.6
***B. saguini* DSM 23967**																								88.5
***B. lemurum* DSM 28807**													86.8	86.9		86.8	89.9	94.6	85.5	87.0				
***B. adolescentis* DSM 20083**																	85.1	84.0						

Based on the neighbour-joining analysis, the novel strains are phylogenetically related to *Bifidobacterium scardovii* and to other novel bifidobacterial species described in non-human primates (Fig. 1). Similar tree topologies were obtained by the maximum-likelihood method (Fig. S2).

Multi-Locus Sequence Analysis (MLSA) is a reliable and robust technique for the identification and classification of bacterial isolates to the species level representing an alternatives to the 16S rRNA gene sequence analysis [17]. Thus, the phylogenetic location of the novel strains was verified by the analysis of six genetic markers (*hsp60, rpoB, dnaG, dnaJ* and *clpC*) which have proven to be discriminative for the classification of the genus *Bifidobacterium* [8,13,38].

Partial *hsp60, rpoB, dnaG, dnaJ* and *clpC* gene sequences, of strains TRE_17^T and TRE_26, TRE_C ^{T} and TRE_M ^{T} and of those of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned by using MAFFT program, at CBRC (http://mafft.cbrc.jp/alignment/software/) [9]. Gblocks version 0.91b, as server tool at Castresana Lab (htpp://molevol.cmima.csic.es/castresana/Gblocks_server.html) was employed to delete poorly aligned positions and divergent regions of DNA alignments, so that they become more suitable for phylogenetic analysis [33].

The concatenation of gene sequences has been shown to be extremely useful in order to infer bacterial phylogeny. Thus, to conduct MLSA, the six housekeeping-gene sequences were concatenated, yielding approximately 4188 bp of sequences. Maximum likelihood method, with Kimura's two-parameter as a substitution model, was used to construct a phylogenetic tree based on the concatenated sequences in MEGA VERSION 6.0. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Fig. 2). The levels of similarity for the eight partial gene sequences obtained from strains TRE_17^T and TRE_26, TRE_C ^{T} and TRE_M ^{T} and their closest relatives are summarized in Table 1.

Despite several efforts using different primer sets and PCR amplification programs (data not shown), the expected fragment for *hsp*60 partial gene sequence was not obtained from strain TRE 26. Therefore, this strain was not included in the MLSA.

Strains TRE_17^T, strain TRE_C^T and TRE_M^T clustered in the *B. scardovii, Bifidobacterium bifidum and Bifidobacterium longum* groups, respectively, thus confirming the 16S rRNA tree topology. Interestingly, all species isolated from ancient non-human primates, viz. *Bifidobacterium aesculapii* DSM 26737^T, *Bifidobacterium biavatii* DSM 23969^T, *Bifidobacterium callitrichos* DSM 23973^T, *Bifidobacterium eulemuris* DSM 100216^T, *Bifidobacterium lemurum* DSM 28807^T, *Bifidobacterium reuterii* DSM 23975^T, *Bifidobacterium stellenboschense* DSM 23968^T and *Bifidobacterium saguini* DSM 23967^T from lemurs and New World Monkeys, are included in these groups while *Bifidobacterium moukalabense* DSM 27321^T, isolated from a wild gorilla, is included in the *Bifidobacterium adolescentis* group.

The G+C content estimation in bacterial chromosomal DNA of strains TRE_17^T, TRE_C ^{T} and TRE_M ^{T} was performed by the DSMZ Identification Service, Braunschweig, Germany. Briefly, DNA was purified on hydroxyl apatit according to the procedure of Cashion *et al.* [3] and was enzymatically hydrolyzed by the method of Mesbah *et al.*[21]. The resulting deoxyribonucleosides were analyzed by HPLC [34]. Type strains TRE_17^T, TRE_C ^{T} and TRE_M ^{T} showed G+C contents of 63.3, 65.9 and 63 mol% G+C, respectively, all values being within the G+C content range of the genus *Bifidobacterium* (52-67 mol%) [1,10] .

Morphological, cultural and biochemical characterization of the isolates according to standard techniques were performed anaerobically at 37 °C unless otherwise stated.

The morphology of cells of strains TRE_17^T, TRE_C ^{T} and TRE_M ^{T}, as revealed by phase-contrast microscopy, and morphological characteristics as determined using a scanning electron microscope (SEM) are shown in Fig. 3. For SEM observations, strains were cultured on TPY broth at 37 °C for 48 h under anaerobic conditions. After culturing, pelleted cells were spotted on grade No.1 qualitative filter paper Whatman and air-dried. Samples were mounted on aluminium stubs with silver glue, and coated with gold–palladium film using an ion-sputtering unit (Emitech K500); observations were made in a Philips 515 SEM at 7–10.0 kV.

Figure 2. Phylogenetic tree based on the concatenate of housekeeping hsp60, rpoB, clpC, dnaG and dnaJ gene sequences showed the relationship of the novel strains TRE_17T, TRE_CT and TRE_MT isolated with closely related species. The tree was constructed by the maximum-likehood method and the sequence of Mycobacterium tuberculosis H37Rv was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap percentages above 50 are given at branching points.

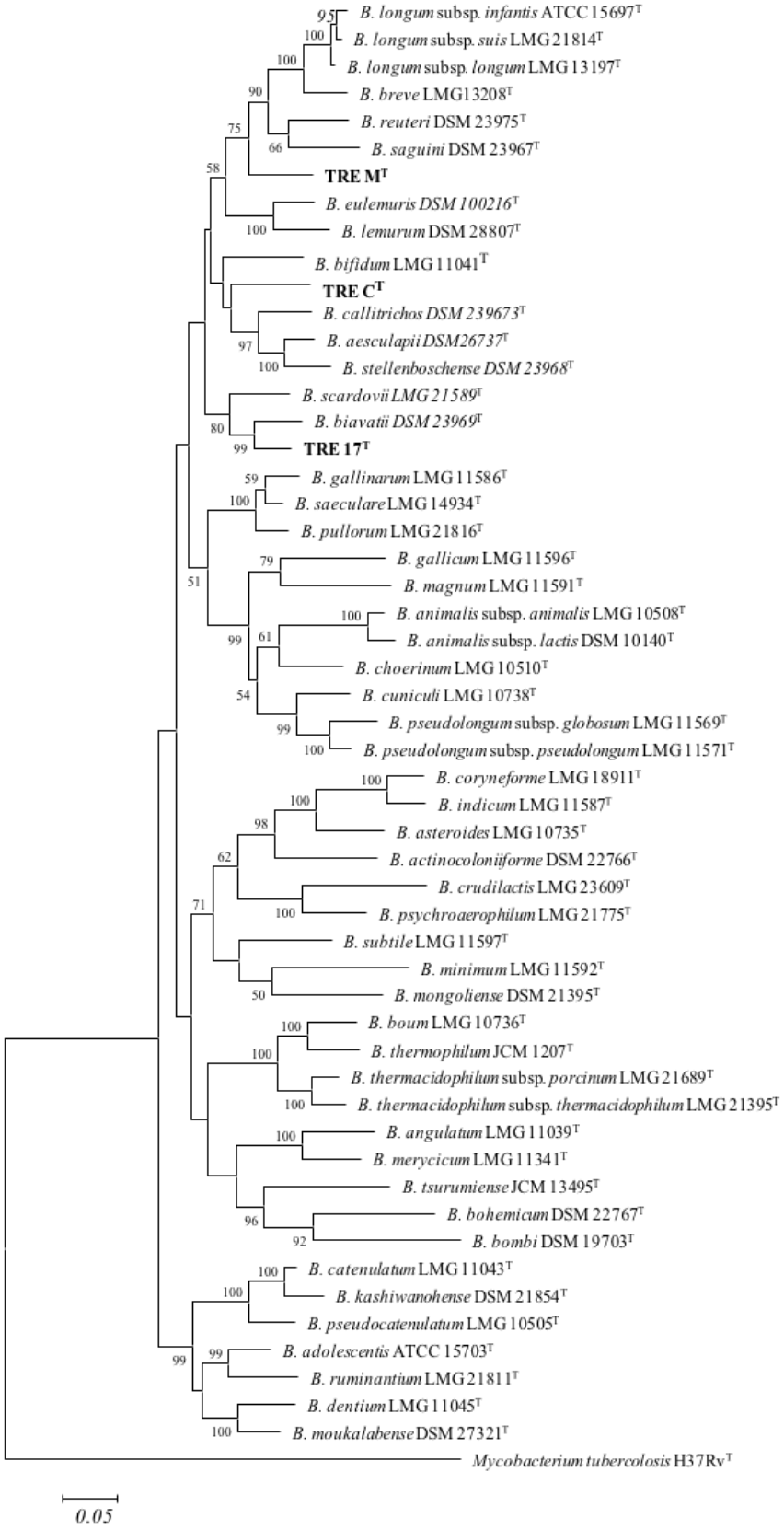
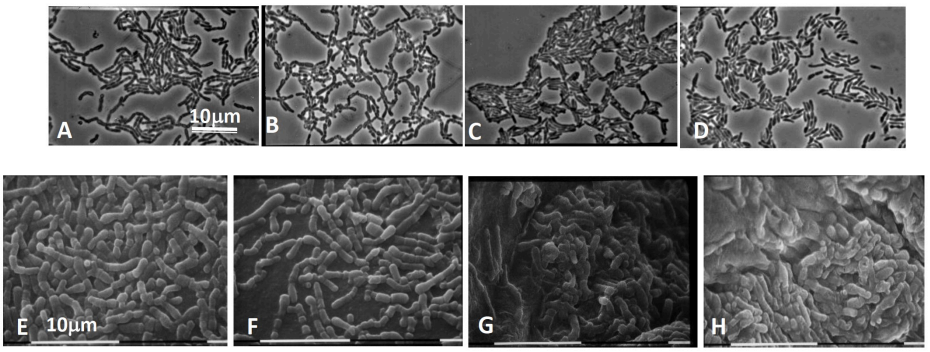


Figure 3. Cellular morphology of cells grown in TPY. Phase-contrast photomicrographs: A) *B. aeriphilum* TRE 17^{T}; B) *B. aeriphilum* TRE 26; C) *B. avesanii* TRE C^{T}; D) *B. ramosum* TRE M^{T}. Scanning electron photomicrograph (SEM): E) *B. aeriphilum* TRE 17$^{T;}$ F) *B. aeriphilum* TRE 26; G) *B. avesanii* TRE C^{T}; H) *B. ramosum* TRE M^{T}.



Optimal growth conditions of each strain were determined in TPY broth after 24 h of incubation at 37°C in anaerobic condition. Growth at 20, 25, 30, 35, 37, 40, 42, 45, 47, 50 and 55°C was tested. Sensitivity to low pH was screened at 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8 values of pH. The ability of the strains to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was also verified in TPY broth after 48 h of incubation at 37 °C. For each strain best growth conditions were obtained in TPY broth pH 6.0 at 40°C. Strains TRE_17^{T}, TRE_C ^{T} and TRE_M ^{T} resulted able to survive and grow in aerobic conditions.
Haemolytic activity was determined in Columbia blood agar (Biolife) at 37°C under anaerobic conditions for 48 h [28].
Gram staining was assessed using cells grown on TPY agar at 37°C under anaerobic conditions for 48 h, with Gram staining individual reagents (Merck Millipore). Motility assay, catalase and oxidase activities have been performed according to Modesto *et al*. [25].
Isolates and related species, viz. *B. scardovii* DSM 13734^{T}*, B. aesculapii* DSM 26737^{T} and *Bifidobacterium bifidum* LMG 13195^{T}, were also investigated for substrates utilization and enzymes production with API 50 CHL and Rapid ID 32 test kits (BioMérieux). Results are summarised in Table 2.

Table 2. Differential phenotypic characteristics of strains TRE_17^{T}, TRE_26, TRE_C ^{T} and TRE_M ^{T}, and their closest relatives *B. scardovii* DSM 13734^{T}, *B. aesculapii* DSM 23937^{T} and *B. saeculare* DSM 6531^{T}.

	TRE_17^{T}	**TRE_26**	**TRE_C ^{T}**	**TRE_M ^{T}**	***B. scardovii* DSM 13734^{T}**	***B. aesculapii* DSM 23937^{T}**	***B. saeculare* DSM 6531 ^{T}**
Enzymatic activity							
Urease	-	-	-	-	w		+
Arginine DiHydrolase	-	-	-	+	-		+
α-galactosidase	+	+	-	-	+		+
β-galactosidase	+	+	+	+	+		-
β-galactosidase 6 phosphate	-	-	-	-	-		+
α-glucosidase	+	+	+	+	+		+
β-glucosidase	w	+	-	-	+		+
α-arabinosidase	+	+	-	+	+		-
β-glucuronidase	-	w	w	-	w		+
N-acetyl-β-glucosaminidase	+	+	-	-	w		+
Mannose fermentation	+	+	+	+	w		+
Raffinose fermentation	+	+	+	+	+		-
Glutamic acid decarboxylase	-	-	-	-	-		-
α-fucosidase	-	w	-	-	+		-
Redaction of nitrates	-	-	-	-	-		+

Indole production	-	-	-	-	-		+
Alkaline phosphatase	+	+	+	+	-		-
Arginine arylamidase	+	w	+	w	+		+
Proline arylamidase	+	+	+	w	-		+
Leucyl glycin arylamidase	+	+	+	w	-		-
Phenylalanine arylamidase	+	+	+	+	+		+
Leucin arylamidase	+	+	+	+	+		-
Pyroglutamic acid arylamidase	+	+	+	+	+		+
Tyrosine arylamidase	+	+	+	+	+		-
Alanine arylamidase	+	+	+	+	w		-
Glycine arylamidase	+	+	+	+	+		-
Histidine arylamidase	+	w	+	+	+		+
Glutamyl glutamic acid arylamidase	+	+	w	+	-		-
Serine arylamidase	+	+	+	+	+		+
Fermentation							
Glicerol	-	-	-	-	w	+	-
Erythritol	-	-	-	-	+	-	-
D-arabinose	-	-	-	-	+	-	-
L-arabinose	-	-	+	+	-	+	+
D-ribose	-	+	-	+	-	+	+
D-xylose	-	-	+	+	-	+	+
L-xylose	-	-	+	-	+	-	-
D-adonitol	-	-	-	-	+	-	-
D-galactose	-	-	w	-	-	-	+
D-glucose	w	+	+	+	+	+	+
D-fructose	+	ww	+	+	-	-	+
D-mannose	+	+	-	-	+	+	+
L-sorbose	-	-	-	-	+	+	-
L-rhamnose	-	-	-	-	+	+	-
Dulciol	+	-	-	-	+	-	-
Inositol	-	-	-	-	+	-	-
D-mannitol	-	-	-	-	-	+	-
D-sorbitol	-	-	-	-	+	-	-
Metil-αD-Mannopyranoside	-	-	-	-	+	-	-
Metil-αD-Glucopyranoside	w	-	-	-	w	-	-
Arbutin	+	+	-	-	-	+	-
Esculin ferric citrate	+	+	-	+	w	+	-
Salicin	w	-	-	-	-	+	-
D-cellobiose	-	+	-	-	+	+	-
D-maltose	+	+	-	-	+	+	+
D-lactose	+	w	-	+	+	+	-
D-melibiose	w	-	-	-	+	w	+
D-saccharose	+	+	+	-	+	+	+
D-threalose	-	-	-	-	w	+	+
Inuline	-	-	-	-	w	w	+
D-melezitose	-	-	-	-	w	+	+
D-raffinose	w	w	+	-	+	+	+
Glycogene	-	-	-	-	-	+	-
Xylitol	-	-	-	-	-	w	+
Gentiobiose	-	-	-	+	+	+	-
D-turanose	+	-	-	-	+	w	-
D-xylose	-	-	-	-	w	+	+
L-arabitol	+	-	-	-	-	-	-
potassium 2-ketogluconate	-	-	w	-	-	w	-

Bifidobacteria and members of related genera posses fructose-6-phosphate phosphoketolase (F6PPK), the enzyme degrading hexose via the F6PPK pathway, which is considered a taxonomic marker for identification of *Bifidobacterium* and related genera [1]. Detection of F6PPK activity was carried out according to the method described by Scardovi [31] and modified by Orban & Patterson [27]. All isolates posses F6PPK activity.

Following the protocol of Schuman [32], the cell-wall murein composition of strains TRE_17^T, TRE_C ^{T} and TRE_M ^{T} was examined by the DSMZ Identification Service.

The total hydrolysat of the peptidoglycan (4N HCl, 16 h at 100 °C) revealed the presence in strain TRE_17^T of muramic acid (Mur) and the amino acids lysine, alanine, glutamic acid and threonine. In addition, the peptide Lys – Thr was detected. After hydrolysis under stronger conditions (6N HCl, 16 h at 150 °C) and derivatization according to Protocol 10, the approximate molar amino acid ratio was determined by gas chromatography / mass spectrometry (320 singlequad, VARIAN) as follows: 1.7 Ala; 0.6 Thr; 1.0 Glu; 0.8 Lys; 0.3 Mur. Two-dimensional TLC of the partial hydrolysate (4N HCl, 100 °C, 45 min) of the peptidoglycan revealed the presence of the peptides L-Ala – D-Glu, L-Lys – D-Ala, L- Lys – L-Thr, D-Ala - L-Lys – L-Thr and L-Ala – D-Ala. On the basis of these results it was concluded that strain TRE_17^T displayed the following peptidoglycan type: A3α L-Lys – L-Thr – L-Ala. The proposed peptidoglycan structure has been detected in lactococci and streptococci but not in bifidobacteria so far.

In strain TRE_C^T the total hydrolysat of the peptidoglycan (4N HCl, 16 h at 100 °C) revealed the presence of muramic acid (Mur) and the amino acids ornithine, lysine, alanine, glutamic acid and serine with the approximate molar amino acid ratio: 1.0 Ala; 0.5 Ser; 2.0 Glu; 0.6 Orn; 0.1 Lys; 0.9 Mur. Chiral analysis of the peptidoglycan amino acids revealed the following molar ratio: 0.2 D-Ala; 0.4 L-Ala; 0.5 D-Ser; 1.0 D-Glu; 0.3 L-Glu; 1.0 L-Orn. Glutamic acid was found to contain a free amino group due to incomplete cross-linkage of the peptidoglycan and therefore represents the *N*-terminus of the interpeptide bridge. Two-dimensional TLC of the partial hydrolysate (4N HCl, 100 °C, 45 min) of the peptidoglycan revealed the presence of the peptides L-Ala – D-Glu, L-Orn (Lys) – D- Ala, L-Orn(Lys) – D-Ser and D-Ala - L-Orn(Lys) – D-Ser. On the basis of these results it was concluded that strain TRE_C^T displayed the following peptidoglycan type: A4β L-Orn (Lys) – D-Ser – D-Glu. This proposed peptidoglycan structure is derived from the peptidoglycan type occurring in *Bifidobacterium boum,* by replacement of most of the lysine residues by ornithine. Possibly D-glutamic acid in the interpeptide bridge is partially replaced by L-glutamic acid.

In strain TRE_M^T the total hydrolysat of the peptidoglycan (4N HCl, 16 h at 100 °C) revealed the presence of muramic acid (Mur) and the amino acids ornithine, alanine, glutamic acid and serine with the following approximate molar amino acid ratio: 2.7 Ala; 1.1 Ser; 1.0 Glu; 0.6 Orn; 0.8 Mur. Chiral analysis of the peptidoglycan amino acids revealed the following molar ratio: 0.4 D-Ala; 1.1 L-Ala; 1.0 L-Ser; 1.0 D-Glu; 2.0 L-Orn. Alanine was found to contain a free amino group due to incomplete cross-linkage of the peptidoglycan and therefore represents the *N*-terminus of the interpeptide bridge. The partial hydrolysate (4N HCl, 100 °C, 45 min) of the peptidoglycan revealed the presence of the peptides L-Ala – D-Glu, L-Orn – D-Ala, L- Orn – L-Ser, D-Ala - L-Orn – L-Ser and L-Ala – D-Ala. On the basis of these results it was concluded that strain TRE_M^T displayed the following peptidoglycan type A3β L-Orn – L-Ser – L-Ala. The proposed peptidoglycan structure has not been detected in any organism so far.

Based on the phylogenetic analyses of the 16S rRNA, *hsp60, rpoB, dnaG, dnaJ* and *clpC* partial gene sequences and according to other data recognized, all studied strains were genetically and phenotypically discernable from each other and from the currently recognized species of bifidobacteria, and thus represent three novel taxa for which the names *Bifidobacterium aeriphilum* sp. nov. (TRE_17^T and TRE_26), *Bifidobacterium avesanii* sp. nov. (TRE_C^T) and *Bifidobacterium ramosum* sp. nov. (TRE_M^T) are proposed.

Description of *Bifidobacterium aeriphilum* sp. nov.

Bifidobacterium aeriphilum (aer'i.phi.lum L. masc. n. aer, aeris air; Gr. adj. philos loving; N.L. neut. adj. aeriphilum air-loving).
Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative, and when growing in TPY broth are rods of various shapes forming a branched structure with 'Y' at the both side. The well isolated colonies grown on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while the embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.0 mm in diameter after 3 days of incubation. Cells can grow under aerophilic and microaerophilic conditions. Cells can grow in the range 25 - 50 °C; no growth occurs at 20 or 56 °C. Strains grow at pH 4.0–7.5. Optimal conditions of growth occur at pH 6 and 40 °C. The optimal conditions of growth occur at pH 7 and at temperature of 42 °C. Using API 50 CHL system acids are produced from D-glucose, D-fructose, D-mannose, dulcitol, arbutin, D-maltose, D-lactose, D-sacccharose, D-turanose and L-arabitol and produced weakly from Metil-α-D-glucopyranoside, salicin, D-melibiose and D-raffinose but not from other carbohydrates in API50CH. Activity was observed for α-galactosidase, β-galactosidase, α-glucosidase, α-arabinosidase, n-acetyl-β-glucosaminidase, alkaline phosphatase, proline arylamidase, leucyl glycol arylamidase, phenylalanine arylamidase, leucin arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Activity was also observed from β-glucosidase, arginine arylamidase, histidine arylamidase, and, weakly, from β-glucuronidase and α-fucosidase. Negative response was showed from urease, arginine, dihydrolase, β-galactosidase 6-phosphate and glutamic acid decarboxylase. No redaction of nitrates was recognized. The peptidoglycan type is A3α L-Lys – L-Thr – L-Ala.
The type strain TRE_17^{T} (=DSM 100689 ^{T} =JCM 30941^{T}) and the reference strain TRE_26 (=DSM 100690 =JCM) 30942 were isolated from the faeces of an adult subject of the cotton top tamarin. The DNA G+C content of the type strain is 63.3 mol%.

Description of *Bifidobacterium avesanii* sp. nov.

Bifidobacterium avesanii (aves'a.nii, N.L. masc. gen. n. *avesanii*, of Avesani, named after Doctor Alberto Avesani, the Founding Father of Parco Natura Viva, Verona North Italy).

Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and when growing in TPY broth under anaerobic condition, are rods of various shapes forming a branched structure with 'Y' at the both side. Well isolated colonies, on the surface of TPY agar plates, reached about 1.5–2.5 mm of diameter after 2 days of incubation under anaerobic conditions and are white, opaque, smooth and circular with entire edges; when embedded, colony shapes are lens or elliptical. Cells can grow under aerophilic and microaerophilic conditions. Growth in TPY broth occurs in the range 25 -50 °C, but not at 20 neither at 56 °C (after 24-48h). Cells can grow in the pH range of 4.0–7.5. Optimal conditions of growth occur at pH 6 and 40 °C.
Cells produce acids from L-arabinose, D-xylose, L-xylose, D-glucose, D-fructose, D-sacccharose and D-raffinose and produced weakly from of D-galactose, potassium 2-ketogluconate and raffinose but not from other carbohydrates in API50CH. Mannose fermentation was recognized using the API Rapid ID32 system and enzymatic activities for β-galactosidase, α-glucosidase, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl glycin arylamidase, phenylalanine arylamidase, leucin arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase,

glycine arylamidase, histidine arylamidase and serine arylamidase were also observed. A weak activity was also recognized for β-glucuronidase and glutamyl glutamic acid arylamidase. No redaction of nitrates was observed. The peptidoglycan type is A4β L-Orn(Lys) – D-Ser – D-Glu.
The type strain TRE_C^T (=DSM 106805^T =JCM 30943) was isolated from the faeces of an adult subject of the cotton top tamarin. The DNA G+C content of the type strain is 65.9 mol%.

Description of *Bifidobacterium ramosum* sp. nov.

Bifidobacterium ramosum (ra'mo.sum, N. masch. *ramus*, neut. adj. *osum*, N.L. neut. adj. *ramosum*, full of boughs, having many branches, branching, branchy).

Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and when growing in TPY broth under anaerobic condition, are rods of various shapes forming a branched structure with 'Y' at the both side. On the surface of TPY agar the colonies reached 1.0–2.5 mm in diameter after 2 days of incubation and are white, opaque, smooth and circular with entire edges; imbedded colonies showed lens or elliptical shapes. Cells can grow under aerophilic and microaerophilic conditions. Growth in TPY broth occurs in the range 25-50 °C; no growth occurs at 20 neither at 56 °C (after 24-48 h). Strains grow in the pH range 4.0–7.5. Optimal conditions of growth occur at pH 6 and 40 °C under both microaerophilic either anaerobic conditions.
Cells produce acids from L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-lactose, and gentiobiose but not from other carbohydrates in API50CH. Esculin is hydrolyzed. Mannose and raffinose fermentation was recognized using the API Rapid ID32 system. Positive enzymatic activity was also observed for arginine, dihydrolase, β-galactosidase, α-glucosidase, α-arabinosidase, alkaline phosphatase, phenylalanine arylamidase, leucin arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase and, weakly, for arginine arylamidase, proline arylamidase, leucyl glycin arylamidase, β-glucosidase, α-galactosidase and β-galactosidase. No activity was showed for urease, β-galactosidase 6 phosphates, β-glucuronidase, n-acetyl-β-glucosaminidase, glutamic acid decarboxylase and α-fucosidase. Nor reduction of nitrates neither production of indole was observed. The peptidoglycan type is A3β L-Orn – L-Ser – L-Ala.
The type strain is TRE_M^T (=DSM 100688^T =JCM 30944^T) was isolated from the faeces of an adult subject of the cotton top tamarin. The G+C content is 63.0%.

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CHAPTER 3

3. BIFIDOBACTERIA IN THE GUT OF PRIMATES

A complex, dynamic and critical, to both health and disease, microbial community inhabits the gastrointestinal tract of primates and its composition and constituents are influenced by multiple internal and external factor, such as host diet, geography, physiology, and disease state (Ochman et al., 2010).

Living in the gut requires that communities of microorganisms show (i) several enzymes to utilize available nutrients; (ii) cell-surface molecular paraphernalia to attach to the specific niches, evade bacteriophages, and appease a reaction-ready immune system; (iii) genetic gadgetry for mutability to stay well adapted; (iv) the ability to avoid washout with a rapidly growth; (v) and stress resistance needed when making the jump to other hosts via a largely dry and toxic "*ex-host"* environment (Ley et al., 2006).

The main topic of ecology biologist is the acquisition of knowledge about how evolutionary and ecological processes generate and maintain biodiversity, while evolutionary biologists focus their attention on the mechanisms controlling rates of evolution and how this influences the phylogenetic relationship among species, ecologists attempt to explain the distribution and abundance of taxa based upon interactions among species and their environment (Lennon and Denef, 2015). Recently, researches get over this distinction integrating ecology and evolutionary in the eco-evolutionary approach.

3.1. PRIMATE PHYLOGENY: APES VERSUS MONKEYS

Humans belong to the biological group known as Primates and are classified within the great apes sub-groups, one of the major in the primate evolutionary tree (Figure 3).

Primates could be ordered in to three different groups: Prosimians, Monkeys and Apes, which contain two sub-groups, lesser and great apes. In the last one, humans, bonobos, common chimpanzee, Western and Eastern gorillas, and Bornean and Sumatran orangutans, which share the 96-99% of DNA each other, are included.

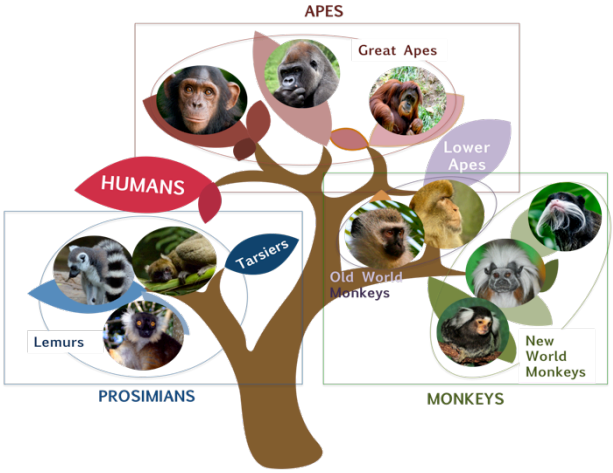

Figure 3. Schematic primate evolutionary tree.

The differentiation between apes and monkey is related to the way of their scientific classification. Indeed, apes and Old World (Africa- and Asia-based) monkeys share common ancestors, but after a certain time in the evolutionary history they took separate branches in the evolutionary tree. New World monkeys belong to a parvorder, known as *Platyrrhini,* the term refers to flat-nosed, while Old World monkeys and apes belong to the parvorder of *Catarrhini*, that means hook-nosed. *Catarrhini* are further differentiated by superfamilies: *Cercopithecoidea* that comprised the Old World monkeys, and *Hominoidea* in which Great Apes and Lesser Apes are classified,

Table 4.

Table 4. Prosimians, monkey and apes phylogeny summary.

PROSIMIANS	**MONKEYS**		**APES**	
	Platyrrhini (parvorder)	*Catarrhini* (parvorder)		
		Cercopithecoidea (superfamily)	*Hominoidea* (superfamily)	
	New World monkeys (group)	Old World monkeys (group)	Lesser Apes (sub-group)	Great Apes (sub-group)
Lemurs, lorises, and tarsiers	Marmosets, tamarins, and capuchins live in South and Central America	Baboons, macaques, and colobus monkeys live in Africa and Asia	Gibbons and siamangs	Humans, gorillas, chimpanzees, orangutans, and bonobos

3.2. **"YOU ARE NOT WHAT YOU EAT"** (OCHMAN ET AL., 2010)

As well documented in literature, the acquisition of a new diet plays a main role in driving the evolution of a species. Not only the individuals of the species are involved, but also the microorganisms that inhabit their gastrointestinal tract.

The digestive tract of mammalians is sterile at the birth and is soon colonized by bacteria that derive from the mother (Favier et al., 2003; Inoue and Ushida, 2003; Tannock et al., 1990).

Ochman *et al.*, in the 2010, investigating the gut micriobial communities arboured by great apes, dimostred that over evolutionary time-scale, the composition of the gut microbiota among great apes species is phylogenetically conserved. Indeed, there is (i) a specie-specific signature of microbial

community structure and (ii) the pattern of relationships among the great apes species, the host phylogeny, shapes the gut microbiota. In conclusion, the authors propose the sentence: "*You are not what you eat*".

3.3. THE MICROBIAL GUT COMMUNITY

Compare to other microbial habitats, the mammals gut is poorly populated by microorganisms reflecting the relative short time that it has been existing as habit, about 100 million years for mammals with placentas, versus more than 3.85 billion of years for the ocean (Murphy et al., 2001). The human gut is inhabited by microorganisms belonging to 9 different divisions of Bacteria: *Firmicutes* and *Bacteroides*, the most represented (about 98% of the totality of Bacteria), *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Verrucomicrobia, Cyanobacteria, Spirochaetes* and *VadinBE97* (Bäckhed et al., 2005; Ley et al., 2006).

3.3.1. FACTORS THAT INFLUENCED THE MICROBIAL GUT DIVERSITY

Although an abundance of microorganisms populate the mammals intestinal tract, at the born they are germ free and the microbes must come from the outside, from the vagina "inoculum" and faeces of their mothers (Mändar and Mikelsaar, 1996). After the first colonization, the host plays a strong role modulating directly the composition of the microbiota.

The microbial diversity in the gut of mammals is forced by different factors: (i) method of colonization, (ii) physical and (iii) chemical environment, and (iv) selective pressure on the host (Ley et al., 2006).

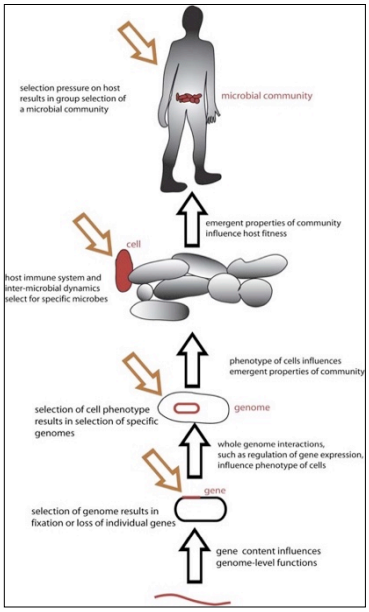

Figure 4. Diagram of the selection pressures in the human-microbial hierarchy. Brown arrows indicate selection pressures and point to the unit under selection (red). Black arrows indicate emergent properties of one level that affect higher levels in the hierarchy.

As explain above, the microorganisms that arbour the mammal gut derives from the mothers so it appears clear how microbial communities that colonized the gut are similar in individuals of a given family. Ley *et al.* (2005) with their work demonstrated that the microbiota is inherited vertically from mothers and it is stable enough over time that kinship relationships are reflected in community composition.

The host and its microbial gut community could be considered as peculiar biological system where each microorganism is under a selection pressure acts by the host on the cell's phenotype and resulting in fixation of gene in the genomes (Ley et al., 2006). The pressure is related to the services that the microbiota should be provided to the host fitness, such as contribution to host nutrition by enhancing the efficacy of energy harvest from ingested food and by synthesizing essential vitamins (Bäckhed et al., 2005). Indeed, as in detail explain by Ley *et al.* (2006), the host does not receive benefits from its microbiota, the entire group is selected against at the death of the host. The authors have been identified two levels of selection to explain the microbial diversity in the human gut:

1. Host level or "top-down" selection, represents the pressure

operating by the host, which favours the stability of the societies and high decree of functional redundant in its encoding genomes.

2. Microbial cells level or "bottom-up" selection, it is the pressure driven by the microorganisms for promoting their functional specialization. This competition between microbes results in selection of genomes with specific genes, metabolic traits.

According to the ecology theory developed by Wilson in the 1975, the community must occasionally change habit and colonize new hosts allowing that the group selection can take place. This transmission affected the scale at which competition between members of the microbiota occurs from local, within the host, to global, between hosts (Griffin et al., 2004).

3.3.2. Co-Evolution Between Host and Microbiota

The term co-evolution (or co-diversification) is used to define the reciprocal adaptation occurring between interacting species (Moran, 2006) and has been hypnotized to occur in species whose (i) parental care enables vertical transmission of whole gut communities and where (ii) the totality of the community properties are able to confer a fitness advantage to the host (Ley et al., 2006): firstly digestion of the food and release of nutrients and energy.

At the birth the gastrointestinal tract of mammals is colonized by outcome microorganisms, and if no subsequent alteration or additional colonization occur, the pattern of constituents and composition of microbiota would co-diversify with, and ultimately mirror, the evolutionary relationship of their host due to parental inheritance (Ochman et al., 2010). Furthermore, microbial community of closed related host species seems to be more functionally interchangeable than the community of distant related host species.

The gut could be considered as an efficient and stable bioreactor, resistant and resilient to the entrance in the environment of sub-population or pathogens (Bäckhed et al., 2005) providing stability to the host/microbiota system. However, several internal and external factors, such as diet, geography, host physiology, disease state and features of the gut, give a contribution to change and define the composition of the microbial community in the gut and causing discordance with the phylogeny of the host (Ochman et al., 2010).

Recently, McKenney *et al.*, (2015) examined the relationship between host lineage, captive diet, and life stage and gut microbiota characteristics in *Varecia variegate*, *Lemur catta* and *Propithecus coquereli*. Results have been revealed community membership and succession patterns consistent with previous studies of human infants. The authors also suggested lemurs as useful model of microbial ecology in the primate gut. Indeed species-specific bacterial diversity signatures appeared correlating to life stage and life history traits. Furthermore, authors delineated a putative core microbiomes, which should be recognised for all three species as lemur approach weaning and adulthood.

3.3.3. Sympatric Animals: Convergent Gut Microbial Communities

In the previous paragraph we discussed about co-evolution between hosts and their gut microbiota. This is probably due both to heritable factors, such as host genetics and the vertical, generation-to-generation transmission of gut microbes (Vaishampayan et al., 2010) and environmental factors, such as host diet and geography distribution (De Filippo et al., 2010; Ley et al., 2008; Turnbaugh et al., 2009; Yatsunenko et al., 2012).

Nevertheless, Moeller *et al.*, in the 2013, found that cause great ape species sampled to date represent populations that are at once phylogenetically, ecologically, and geographically distinct, it has not been possible to separate the relative influences of heritable and environmental factors on the evolution of the great ape gut microbiota. Comparing allopatric and sympatric host great ape hosts, the authors pointed out that until 6 million years ago, when Gorillas has been not yet diverged from the lineage leading to human, chimpanzees and bonobos, and they come into secondary contact in throughout equatorial Africa, they experienced dietary convergence in addition to shared geography (Stanford, 2006; Yamagiwa and Basabose, 2006), but do not mingle or interbreed, maintaining their phylogenetic distinctiveness. Despite this, researchers found that while hosts of different species generally always maintain distinct gut microbiota (even when living in sympatry), the gut microbiota of sympatric *Pan* and *Gorilla* share significantly more bacterial phylotypes than do those of allopatric *Pan* and *Gorilla*. In addition, based on the specific patterns of phylotype sharing, they were able to demonstrate the presence of a history of gut bacteria transfer between the two host species, with chimpanzees acquiring bacteria from sympatric gorillas.

3.4. MICROBIAL COMMUNITY DIVERSITY AND ABUNDANCE ANALYSIS

Deepen the complexity of bacterial diversity is of particular importance because bacteria may well comprise the majority of earth's biodiversity and mediate critical ecosystem processes (Cavigelli and Robertson, 2000; Torsvik et al., 2002).

The microbial biodiversity describes complexity and variability among microorganisms at different levels of biological organization, including genes, species, ecosystems, evolutionary and functional processes that link them (www.for. gov.bc.ca/pab.publctns/glossary/b.htm).

Nowadays, techniques to characterize and classify microbial communities by cultivation methods have switched to the molecular and genetic level, thus as reported by Muyzer *et al.*, 1993. However, both culture dependent and independent methods contains certain limitations and none of them is perfect regarding the identification of an unknown bacterium (Das et al., 2014); in Figure 5 the taxonomic resolutions of the currently used techniques is showed.

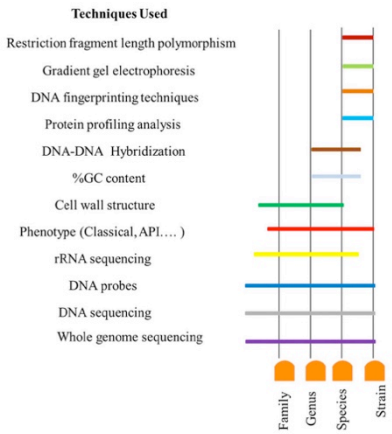

Figure 5. Taxonomic resolutions of the currently used techniques (Das et al., 2014).

Cultivation-based techniques allowed merely a glimpse of microbial diversity as only an estimated 1% of the naturally occurring bacteria isolated and characterized so far. In this view a

polyphasic approach involving a combination of molecular biology techniques and conventional microbiological methods seems necessary for a better understanding (Das et al., 2014).

3.4.1. MOLECULAR APPROACHES

Taxonomists have been developed different molecular approaches for the study of microbial diversity in an environmental sample, which provide rapid profiling of microbial communities offering information about phylogenetic groups present.

A possible classification of these techniques should be based on different type of polymerase chain reaction (PCR).

a) PCR-independent approaches

Several approaches have been developed to study the microbial community. PCR-indipendent methods include guanine and cytosine content (G+C), DNA-reassociation kinetics and DNA-DNA hybridization (DDH) and reverse sample genome probing (RSGP).

Guanine plus cytosine (G+C) **content** is based on the difference G+C content of DNA to measure bacterial diversity in the crude DNA extracted from the sample. Nusslein & Tiedje (1999) reported that microorganisms differ in their G+C content and that taxonomically related groups differ only by 3 and 5 %. The measure is performed after fractionation of total community DNA by density gradient centrifugation based on G+C content; the total community DNA is physically separated into highly purified fractions, each representing a different G+C content (Das et al., 2014).

Whole-genome **DNA-DNA hybridization** offers true genome-wide comparison between organisms (Das et al., 2014). Although DDH techniques have been originally developed for pure culture comparisons, they have been modified for use in whole microbial community analysis. In DDH technique, total community DNA extracted from an environmental sample is denatured and then incubated under conditions that allow them to hybridize or reassociate, **DNA-reassociation kinetic** method.

Analyses of the microbial community composition, considering the most dominant culturable species, should be carried out by reverse sample genome probing (RSGP). The method is useful when samples are characterized by low diversity, but several molecular biologists face difficulty while assessing community composition of diverse habitats (Green and Voordouw, 2003). RSGP includes four steps: (i) isolation of genomic DNA from pure cultures, (ii) cross-hybridization testing to obtain DNA fragments with less than 70 % cross-hybridization, (iii) preparation of genome arrays on a solid support, and (iv) random labeling of a defined mixture of total community DNA and internal standard (Das et al., 2014).

b) PCR-dependent approaches

PCR-based molecular methods provide a fast and sensitive alternative to conventional culture techniques (Agrawal et al., 2010). Mainly, molecular 16S rDNA-based PCR techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphisms (SSCPs), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphisms (T-RFLPs) and ribosomal intergenic spacer analysis (RISA) can provide detailed information about community structure of an ecosystem in terms of richness, evenness and composition and can be used to compare different species present in a (Rawat and Johri, 2014).

The **16S rDNA** gene regions, which are highly conserved within closely related taxa (Santos and Ochman, 2004), have been used as a phylogenetic marker for classification of bacteria into different taxa (Singh et al., 2011). Avoiding limitations of cultivability, the categorization of the 16S rDNA gene from the community DNA of environmental samples has become wide popular as an alternative to characterise microbial communities directly providing information on phylogenetic diversity (Zhou et al., 1997). The use of this technique required cloning and sequencing strategies, which are cost, time and labour consuming for the monitoring a large number of samples. I

In the 1993, Muyzer *et al.* introduced the denaturing gradient gel electrophoresis (**DGGE**) of the PCR amplified DNA fragments in the microbial ecology to study the structural diversity of microbial communities. DGGE represents a consolidate approach for diversity study that overcomes the disadvantages in cloning and sequencing of the DNA fragments (Singh et al., 2011).

ARDRA is based on DNA sequence variations present in PCR-amplified 16S rRNA genes the PCR amplicon from environmental DNA is digested with tetracutter restriction endonucleases, such as *AluI* and *HaeIII*, and restricted fragments are resolved on agarose or polyacrylamide gels (Agrawal et al., 2010). This method provides little or no information about the type of microorganisms present in the sample, but it is still useful for rapid monitoring of microbial communities over time, or to compare microbial diversity in response to changing environmental conditions (Agrawal *et al.*, 2010). ARDRA is also used as sensitive technique with high resolution providing reliable genotypic characterization at the community level of compost bacteria (Heyndrickx et al., 1996).

Repetitive element sequence-based polymerase chain reaction (**rep-PCR**) fingerprinting represents a genotypic technique using outwardly facing oligonucleotide PCR primers complementary to interspersed repetitive sequences, such as evolutionarily conserved repetitive sequences are BOX, ERIC, REP and $(GTG)_5$, which enable the amplification of differently sized DNA fragments lying between these elements (Masco et al., 2007). Rep-PCR fingerprinting is a valuable tool for classifying and typing of a wide range of Gram-negative and sever- al Gram-positive genera (Versalovic et al., 1994). Indeed, the distribution of these repetitive sequences (BOX and ERIC) as nearly a true reflection of genomic structure and amplification of inter- REP elements often detects similarities in a given group of bacteria (Agrawal et al., 2015). BOX elements are repetitive sequences randomly located within the whole genome and by using BOX primers the amplification of genomic regions between the two BOX elements should be performed. (Masco et al., 2003) testing different rep-PCR methods, has been reported the BOXA1R primer has the most suitable rep primer for the identification of bifidobacteria. Enterobacterial repetitive intergenic consensus (ERIC) sequences are short interspersed repetitive elements found in the genome of eubacteria (Gillings and Holley, 1997) and distributed throughout extragenic regions of the genomes of many gram negative enteric bacteria and closely related phyla (Versalovic et al., 1994). Cause their unique location, ERIC elements in bacterial genomes allows discrimination at genus, species, and even strain level based on the electrophoretic pattern of amplification products (de Bruijn, 1992). Selective amplification of ERIC elements using oligonucleotide primers generates amplicons of varying sizes, ranging from 50 to 3000 bp, which collectively constitute a DNA fingerprint (Di Giovanni et al., 1999a). Comparative studies of electrophoretic fingerprints are used for identification, discrimination and classification of bacterial strains or communities (Ben Amor et al., 2007; de Bruijn, 1992; Di Giovanni et al., 1999b). There are only few literature information on application of such molecular based techniques (BOX- and ERIC- PCR) in studying the microbial communities in environmental sample (Cifuentes et al., 2000; Dunbar et al., 2000; Ennahar et al., 2003; Hobel et al., 2005; Singh et al., 2011).

3.5. BIFIDOBACTERIA IN THE GUT OF PRIMATES

Bifidobacteria are distributed in six ecological niches, encompassing the human intestine, oral cavity, insect and animal intestine, sewage, blood and food (Ventura et al., 2012, 2007). All these niches are directly or indirectly linked to the human/animal intestinal environment (Ventura et al., 2014).

Latest studies have been demonstrated that wild chimpanzees (*Pan troglodytes verus*) harboured *Bifidobacterium angulatum*-like bacteria as a common component of their intestinal microbiota (Ushida *et al.,* 2010); while a novel species, *Bifidobacterium moukalabense,* was isolated from the faeces of a wild lowland gorilla in Moukalaba-Doudou National Park (MDNP) in Gabon (Tsuchida et al., 2014).

In our laboratory, *Bifidobacterium dentium* and *Bifidobacterium adolescentis* were isolated from faeces of chimpanzees (*Pan troglodytes*) and orangutans (*Pongo pygmaeus*) respectively, housed at Borås Djurpark, a zoo in the northern part of central Borås (D'Aimmo et al., 2012a). Thus, the presence of bifidobacteria associated with non-human great apes may be suggested.

The study of diversity of cultivable bifidobacteria in gut of non-human primates is a very interesting topic due to their close evolutionary level to human beings. Moreover, such investigation should allow isolation and characterization of new *Bifidobacterium* species or strains that could have interest also in probiotic area.

3.6. CASE OF STUDY 1. THE CULTIVABLE AND UNCULTIVABLE BIFIDOBACTERIA HARBORED BY PRIMATES AND A COPHYLOGENETIC ANALYSIS

Refers to **PAPER 4** and **DRAFT 2, 3, 4**.

The microbial community living in the gut play a main role contributing to host nutrition, health and behaviour. The community diversity differs according to host phylogeny and the relationship between host and gut microorganism is driven by their co-evolution. This is a current hot topic, even if our existing understanding is limited (Amato, 2013).

3.6.1. AIM OF THIS WORK

Second aim of our research is to deepen the bifidobacteria diversity of primates to increase our knowledge about their ecology and to investigate the co-evolution with host by exploring both the cultivable (**PAPER 4** and **DRAFT 2**) and the uncultivable bifidobacteria diversity and abundance (**DRAFT 3**). Moreover, with our analyses, we would confirm the hypothesis that close host species are functionally interchangeable.

3.6.2. MATERIALS AND METHODS

Materials and Methods used for isolation, grouping, identification and quantification of cultivable bifidobacteria strains from primates are extended explained in **PAPER 2, 4, 6** (common marmoset), **PAPER 3** (ring-tail lemur), **PAPER 5** (black lemur) and **DRAFT 1, 2** (emperor and cotton top tamarin). The same approach was also utilized for the analysis of fresh fecal samples from grivet, Barbary macaque and Lac Alaotra Bamboo lemur.

For a quick explanation, Materials and methods of this section were divided in a) cultivable and b) uncultivable approach.

a) Cultivable approach

All isolates were obtained from fresh fecal samples of primates.

Briefly, after serial dilution of fecal sample in peptone water supplemented with cysteine hydrochloride, 1 ml aliquot of each one was inoculated onto selective media, TPY added with mupirocin and/or TOS and/or MRS added with cysteine hydrochloride. After 24/48 hours of incubation, 20-50 colonies were randomly picked from each fecal sample. Enumerations were performed by plate count. Isolates were tested for fructose-6-phosphate phosphoketolase (F6PPK) activity, the key enzyme for the family *Bifidobacteriaceae*. Genomic DNA was extracted and to group clones a cluster analysis on the BOX DNA-fingerprinting from each isolate was carried out using the clustering-based peak alignment algorithm (Ishii *et al.*, 2009) for RStudio program (**PAPER 6**) and Gel Compare II software (**DRAFT 2**).

The identification of representatives for each clusters, described in **PAPER 4** was performed by using the RFLP-PCR method by Baffoni *et al.* (2013) updated as described in **PAPER 1.** For unrecognized profiles, the sequencing of the partial 16S rRNA genes was performed for the identification.

Identification of representatives for each clusters of strains isolated in **DRAFT 2** was directly performed through the sequencing of the partial 16S rRNA genes.

b) Uncultivable approach

This work would represent an initial study to pave the way for identify methods for the quick and low cost study of the community diversity in faecal samples.

Uncultivable microorganisms and bifidobacteria were detected in all faecal samples; diversity was analyzed by rep-PCR, such as BOX- and ERIC-PCR, and ARDRA, through two double digestion performed on 16S rRNA gene amplified by using universal and bifidobacteria genus-specific primers, while quantification of bifidobacteria, lactobacilli and enterobacteria was performed by Real-Time PCR.

3.6.3. RESULTS AND DISCUSSIONS

Results from both the cultivable and uncultivable approach allowed us a more knowledge about the diversity of bifidobacteria and abundance of bifidobacteria, lactobacilli and eneterobacteria in lemurs and new world monkeys.

a) Cultivable approach

Results and Discussions are extensively described in **PAPER 4** and **DRAFT 2**.

The isolates obtained offers a wider overview on the distribution of bifidobacteria in five different species of non-human primates (*Callithrix jacchus*, *Saguinus oedipus, Saguinus imperator*, *Lemur catta*, and *Eulemur macaco*) not already studied (Table 5).

		PROSIMINAS Lemurs		**MONKEYS** New World Monkeys				**APES** Great Apes		
References	**Bifidobacteria Species**	**ring-tailed lemur**	**black lemur**	**common marmoset**	**top-cotton tamarin**	**red-handed tamarin**	**emperor tamarin**	**orangu-tan**	**gorilla**	**chimpanzee**
D'Aimmo et al., 2012	*B. adolescentis*							X		
	B. angulatum									X
Tsuchida et al., 2014	*B. moukalabense*								X	
D'Aimmo et al., 2012	*B. dentium*									X
Endo et al., 2012	*B. biavatii*					**X**	**X**			
	B. saguini					**X**	**X**			
	B. stellenboschense				**X**	**X**				
	B. callithricos			**X**	**X**					
	B. reuteri			X						
PAPER 2	*B. aesculapii*			X						
PAPER 4, 6	*B. myosotis*			**X**	**X**					
	B. tissieri			**X**			**X**			
	B. hapali			X						
PAPER 6	MRM_8.19			X						
	MRM_9.3			X						
DRAFT 1, 2	*B. aeriphylum*				**X**		**X**			
	B. avesanii				X					
	B. ramosum				X					
	TRE_D				**X**		**X**			
PAPER 3	*B. lemurum*	**X**	**X**							
PAPER 5	*B. eulemuris*		X							
DRAFT 4	3 putative sp. nov				X					
	4 putative sp. nov						X			

Table 5. Summary about associations between bifidobacteria species and primate hosts. X in bold highlight bifidobateria species shared by different primate hosts.

Several strains belonging to unknown species in the *Bifidobacterium* genus have been detected. In particular, we found a very high diversity at species level in all the new world monkeys sampled. In particular, babies of common marmoset harboured eight different species, while the adult subject of cotton-top and emperor tamarin are also very rich, with 10 different species for each non-human primate species. According to our data, lemurs and great apes seems to affect by a very low diversity, harbouring only one or two species in their gut.

Considering all the associations, a hypothesis about the relationship between bifidobacteria and non-human primates host can be proposed. Although more investigations are needed to verify and support our hypothesis, we could suggest the existence of a "core" of bifidobacteria for each primate host group. This "core" of species may be closed related to its host, showing a strong species-specificity. On the other hand, other species show a wide host-diffusion and they are shared by different primates belonging to the same group.

Based on this hypothesis, *B. eulemuris* should be considered a representative of the core microbiota of the black lemur, while *B. lemurum*, isolated from both ring tail and black lemur, may to be less host-specific, even if no diffusion of this species out from the lemurs group was found. *B. reuteri, B. aesculapii* and *B. hapali* belong to the "core" microbiota of baby common marmoset; *B.*

avesanii, B. ramosum and 6 putative novel species may be *S. oedipus* host-specific and *S. imperator* could harbour four close related bifidobacteria species. Marmoset and the three species of tamarin, included in the New world monkey group, share some bifidobacteria (*B. aeriphilum, B. biavatii, B. myosotis, B. saguini, B. stellenboschense, B. tissieri* and strain MRM_8.19), which are not yet recognized in other groups, such as lemurs and great apes.

b) Uncultivable approach

Results and Discussions are extensively described in **DRAFT 3**.

Using conventional, rapid and low cost techniques, such as ARDRA, rep-PCRs (ERIC- and BOX-PCR) and Real-Time PCR, we deepen the microbial community diversity and abundance in primate host species at different evolutionary scale.

Regarding ARDRA, our work underlined the potential of the restriction analyses on the bifidobacteria 16S rRNA partial gene sequence; indeed, it is able to detect *Bifidobacterium* spp. in the total microbial DNA from faecal sample and, with modifications regarding the enzymes, seem able to distinguish the harboured bifidobacteria at the species level. The method should be improved and enzymes with higher discrimination power should be recognized if the total microbial 16S rRNA gene is considered.

Rep-PCR methods appear the best method to have a look on the global community diversity, even if no additional information about family, genus or species can be obtained. Cluster analysis on both single and consensus fingerprinting (BOX and ERIC) seem to not reflect the phylogenetic history of the host and in some case appear to be affected by individual diversity.

The quantification of the three main microbial groups, bifidobacteria, lactobacilli and enterobacteria was performed using the Real Time PCR. Our primary results may suggest a low presence of bifidobacteria in evolutionary old primates, such as lemurs and old world monkey, compared to those in more evolute species, such as *Saguinus* spp. and *C. jacchus*. Additionally, in common marmoset and Barbary macaques, the bifidobacteria concentration in adults and baby seem to be the opposite in respect to humans. Differently from bifidobacteria, the amount of lactobacilli in faeces of common marmoset seem not to change according to the age, even if more data should be collected to support the hypothesis. Indeed, in Barbary macaques, lactobacilli and enterobacteria occurance seem to mirror the same trend of bifidobacteria increasing from baby to adult subjects. Results from our study did not support a correlation between the abundance of positive (bifidobacteria and lactobacilli) and negative (enterobacteria) gut-microorganisms (enterobacteria).

Further and depth studies are needed to support the hypothesis of variation in the amount of the main microbial groups from baby to adults and to verify if a correlation between bifidobacteria and lactobacilli and enterobacteria could subsist.

3.7. CASE OF STUDY 2. COPHYLOGENETIC ANALYSIS BETWEEN BIFDOBACTERIA AND PRIMATE HOSTS

Refers to **DRAFT 4.**

Previous studies hypothesize a co-evolution between bifidobacteria and their hosts (Ventura et al., 2012). Based on the results concerning the distribution of bifidobacteria species and isolated strains and considering the literature actually available about the presence of these microorganisms in

primate host, a cophylogenetic study between host and related bifidobacteia (**DRAFT 4**) was carried out.

In the last decades, several bioinformatic tools have been developed for the cophylogenetic analysis, mainly based on phylogenetic trees of both the host and the associated bacteria, and respective links.

3.7.1. AIM OF THIS WORK

To summarize all the data from the present study about the distribution of bifidobacteria species in sampled primates and from the literature about bifidobacterial species occurance in primates, aim of this section is to verify if there is possible to show a co-evolution between primates and bifidobacterial species or if other evolutionary events, such as duplication, host switch losses, failure to divergence, should be considered.

3.7.2. MATERIALS AND METHODS

In the **Materials and Methods** section of the **DRAFT 4** the methods and analysis performed have been described.

Briefly, the cophylogenetic analysis was carried out by using different bioinformatics tools. Several methods were developed to test host-microorganism co-evolution and could be classified in two main groups: i) tree-(topologies-) based programs, such as TreeMap, Jane4 and Core-PA, and ii) global-fit programs, such as PACo and CopyCat.

The cophylogenetic analysis of ten primate hosts species, black and ring tailed lemurs, common marmoset, cotton-top, emperor and red-handed tamarins, chimpanzee, orangutan, gorilla and humans, and 38 associated bifidobacterial species was evaluated by using all the programs in the list. For the phylogenetic tree reconstruction of primates, a web-server was employed, while for bifidobacteria, tree was build on the 16S rRNA gene sequence of bifidobacteria species with primate origin retrieved from the NCBI database and the gene sequences from additional strains amplified in our laboratory.

3.7.3. RESULTS AND DISCUSSIONS

Results and Discussions have been described in **DRAFT 4**.

The cophylogenetic analysis was performed on ten host species and 38 strain and species belonging to the genus *Bifidobacterium*. Host and microorganism trees and range distribution information were used as input data for the following cophylogenetic analysis by using i) topology-based programs (TreeMap3, Jane 4 and Core-PA), ii) event-based programs (PaCo and CopyCat).

From TreeMap3 only the tanglegram, representing the association between bifidobacteria and primate host, was obtained. Due to the complexity of our issue, the program fails in the analysis and does not give additional information about alternative event reconstructions. Thus we used Jane4, an alternative program, able to analyses complex data. Despite not significant congruence between tree topologies was found by the event-based analysis performed in Jane4, due to duplication, occasional host switching and possible failure to speciate events by bifidobacteria, Core-PA offers an opposite scenario. The program was able to hypothize different scenarios with more cospeciation events, even if host-switch and duplication are confirmed as the main. Global-fit methods statistically support a

global cospeciation between host and bifidobacteria, but not all the individual link in the host-bifidobacteria association seems to be significant.

Generally, all the programs used for the cophylogenetic analysis were able to identify a sub-system represented by *Saguinus* spp. and associated bifidobacteria, which is characterized by strong and statistically significant links.

PAPER 6

Michelini, S., Modesto, M., Oki, K., Stenico, V., Stefanini, I., Biavati, B., Watanabe, K., Ferrara, A. & Mattarelli, P. (2015). Isolation and identification of cultivable *Bifidobacterium* spp. from the faeces of 5 baby common marmosets (*Callithrix jacchus* L.). *Anaerobe*, 33, 101-104. doi: 10.1016/j.anaerobe.2015.03.001.

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Note

Isolation and identification of cultivable *Bifidobacterium* spp. from the faeces of 5 baby common marmosets (*Callithrix jacchus* L.)

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ABSTRACT

Ninety-two bifidobacterial strains were obtained from the faeces of 5 baby common marmosets, three known species *Bifidobacterium aesculapii*, *Bifidobacterium callithricos* and *Bifidobacterium reuteri* and 4 novel putative bifidobacterial species were retrieved. The occurrence of bifidobacteria in non-human primate babies is described for the first time.

The gastrointestinal tract of animals and humans is inhabited by complex communities of microorganisms that differ from individual to individual and among the animal species themselves. Bifidobacteria are often associated with health-promoting effects, and are normally found in the gut of animals with host–species-specificity [1]. In humans, the distribution of bifidobacterial species in adults and infants has been found to differ. In recent years there has been important progress in gut microbiota studies based on culture independent techniques, however only culture dependent techniques can be used when the aim is to identify and characterize isolates. Unlike studies of human gut microbiota, those addressing the bifidobacteria of non-human primates are very few: *Bifidobacterium angulatum* and *Bifidobacterium moukalabense*, isolated from wild chimpanzee [2,3]; *Bifidobacterium dentium* and *Bifidobacterium adolescentis* from chimpanzees and orangutan [4]; *Bifidobacterium aesculapii*, *Bifidobacterium callithricos* and *Bifidobacterium reuteri* from common marmoset (*Callithrix jacchus*) [5,6] and *Bifidobacterium saguini*, *Bifidobacterium biavatii* and *Bifidobacterium stellenboschense* from red-handed tamarind [6]. The aim of the present work was to characterize the bifidobacterial cultivable microbiota of common marmoset babies, a completely unknown habitat. The study animals were 5 baby common marmoset subjects (Table 1) kept in Aptuit, Verona, Italy. All had been weaned, and were fed a pelletted diet supplemented with fresh fruit, cranberry juice, biscuits, eggs, bread, milk, muesli and arabic gum (or acacia gum). All were free from intestinal infections, and no antibiotics or probiotics had been administered for two months prior to the beginning of faecal sampling by means of fresh rectal swabs. Isolates were obtained from the faecal samples according to Modesto et al. [5], randomly picking about 15–20 colonies from each faecal sample. The isolates were subcultured, maintained and tested for fructose-6-phosphate phosphoketolase (F6PPK) activity, the key enzyme for the family *Bifidobacteriaceae*, according to Orban and Patterson [7]. DNA was extracted as previously described by Modesto et al. [5]. For isolate discrimination, BOX-PCR with the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) was performed according to Masco et al. [8].

Hierarchical cluster analysis on the BOX-PCR DNA fingerprints was performed using the clustering-based peak alignment algorithm developed by Ishii et al. [9] for the RStudio program (http://www.rstudio.com/). Similarity analysis was carried out with the Pearson product–moment correlation coefficient (PCC) and cluster analysis by UPGMA, the unweighted pair group method with arithmetic mean. The “approximately unbiased” p-values (*au*) for

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Table 1
Reference number of sampled animals, gender, average viable counts obtained on mTPY. Agar and total number of isolates for each sampled subject. Bifidobacterial counts were expressed aslog10 CFU/g of faecal sample.

Reference number of animal	Gender	Age (months)	Avg. of viable counts (log_{10} CFU/g faecal sample)	Standard deviation	Number of isolates
5	Female	3	9.2	0.062	20
6[a]	Female	10	9.3	0.059	15
7	Female	4	9.2	0.0456	19
8[a]	Female	10	8.8	0.0739	20
9	Male	4	8.4	0.146	18

[a] Subject 6 and 8 are twin.

each cluster were calculated by multiscale bootstrapping with 1000 replications; also the "bootstrap probability" (BP) value was calculated. Bifidobacteria isolated from a single animal source with a similarity coefficient of 97% (*au* p-value) were considered members of the same cluster [P1].

Representative isolates were identified according to the *hsp60* RFLP-PCR method developed by Baffoni et al. [10] and Stenico et al. [11] allowing a rapid identification at the species level of 36 *Bifidobacterium* spp. A 16S rRNA gene sequence analysis was carried out for isolates with unknown *hsp60* RFLP-PCR profiles. Amplification of template DNA was performed by PCR using the primer pair BI8F (5′-GGGTTYCGATTCTGGCTCAGGATG-3′) and 15R (5′-AAGGAGGTGATCCARCCGCA-3′) according to Miyake et al. [12]. PCR products were purified with NucleoSpin (Macherey–Nagel GmbH & Co. KG, Germany) and directly sequenced by Eurofins MWG Operon Biotech (Germany). About 1360 bp of the 16S rRNA were used for BLAST analysis on GenBank (http://blast.ncbi.nlm.nih.gov) and the pairwise nucleotide sequence similarity values were calculated using EzTaxon server (http://www.eztaxon.org).

All 5 baby marmosets resulted positive for bifidobacteria, with high cell numbers per gram of faeces (Table 1). A total of 92 isolates, all shown to be Gram-positive on staining, catalase negative and F6PPK positive, were obtained (Table 1).

BOX-PCR fingerprints were obtained from all isolates. Fig. 1 shows the distinct dendrograms from the cluster analysis performed on the isolates of each animal. Different isolates were grouped (graphically enclosed in a rectangle) in the same cluster when an *au* p-value higher than 97% was detected. In each animal, a number of clusters ranging from 4 to 8 was detected (Fig. 1).

One representative strain from each cluster of each subject was selected (Fig. 1, strain number highlighted in a circle), and a total of 33 strains were further characterized. The 33 representative isolates were analyzed by means of *hsp60* RFLP-PCR [10] (Fig. 1S). This analysis grouped 33 isolates into 12 distinct *hsp60* RFLP-PCR profiles, three of which were assigned to *B. aesculapii*, *B. callithricos* and *B. reuteri*, while the patterns produced by the others could not be attributed to any known bifidobacterial species (Table 2).

Furthermore, strains MRM 8.14, MRM 9.6 and MRM 9.14 seemed

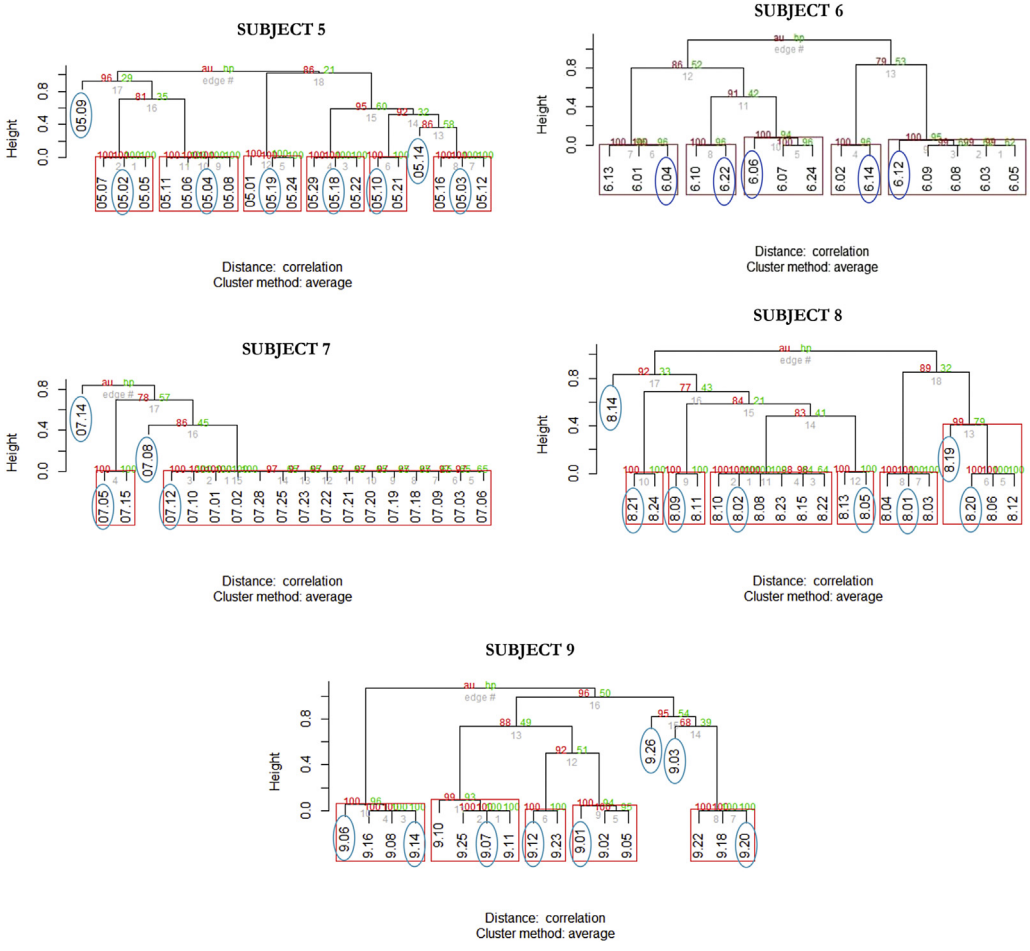

Fig. 1. Hierarchical clustering analysis of the strains isolated. For each sampled subject 5, 6, 7, 8 and 9 a distinct dendrogram was obtained from the elaboration of the BOX-PCR DNA fingerprints of all strains. *Au*, in red, is the "approximately unbiased" *p*-value calculated by multiscale bootstrap resampling (1000 replications); *BP*, in green, refers to the "bootstrap probability" value and edge indicates the clusters. Clusters with *p*-value higher than 97% are indicated in rectangles, and representative strains in circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
RFLP-PCR profiles, occurrence, total number of isolates and relative percentage for each bifidobacterial known species and unknown groups in the five subjects sampled.

Representative strains	Species	RFLP-PCR profile (bp)[a]	Occurrence	Number of isolates
DSMZ 26737^T	*B. aesculapii*	16 – 22 – 31 – 42 – 59 – 123–139 – 158[b]	5/5	28 (30.4%)
DSMZ 23973^T	*B. callithricos*	16 – 22 – 31 – 59 – 462[b]	4/5	26 (28.2%)
DSMZ 23975^T	*B. reuteri*	53 – 59 – 139 – 339[b]	4/5	12 (13.0%)
Groups				
MRM 5.9	MRM_A	25 – 34 – 65 – 126 – 190	1/5	1 (1.0%)
MRM 5.10,0	MRM_B	34 – 47 – 83 – 130 – 150	3/5	8 (8.6%)
MRM 5.18	MRM_C	25 – 35 – 66 – 86 – 118 – 257	3/5	8 (8.6%)
MRM 6.22	MRM_D	80 – 124 – 135 – 270	1/5	2 (2.1%)
MRM 8.19	MRM_E	20 – 65 – 82 – 115 – 120 – 190	1/5	1 (1.0%)
MRM 9.3	MRM_F	109 – 120 – 270 – 510	1/5	1 (1.0%)
MRM 8.14	MRM_G	NF	1/5	1 (1.0%)
MRM 9.6	MRM_H	NF	1/5	1 (1.0%)
MRM 9.14	MRM_I	NF	1/5	3 (3.2%)
				92

[a] In this study also RFLP-PCR profile (3 – 4 – 9 – 81 – 121 – 158 – 172 bp) of *B. moukalabense* DSMZ 23975^T has been described.
[b] Data from Stenico et al. [11]; NF, no fragment; in bold, strains studied for 16S rRNA gene analysis.

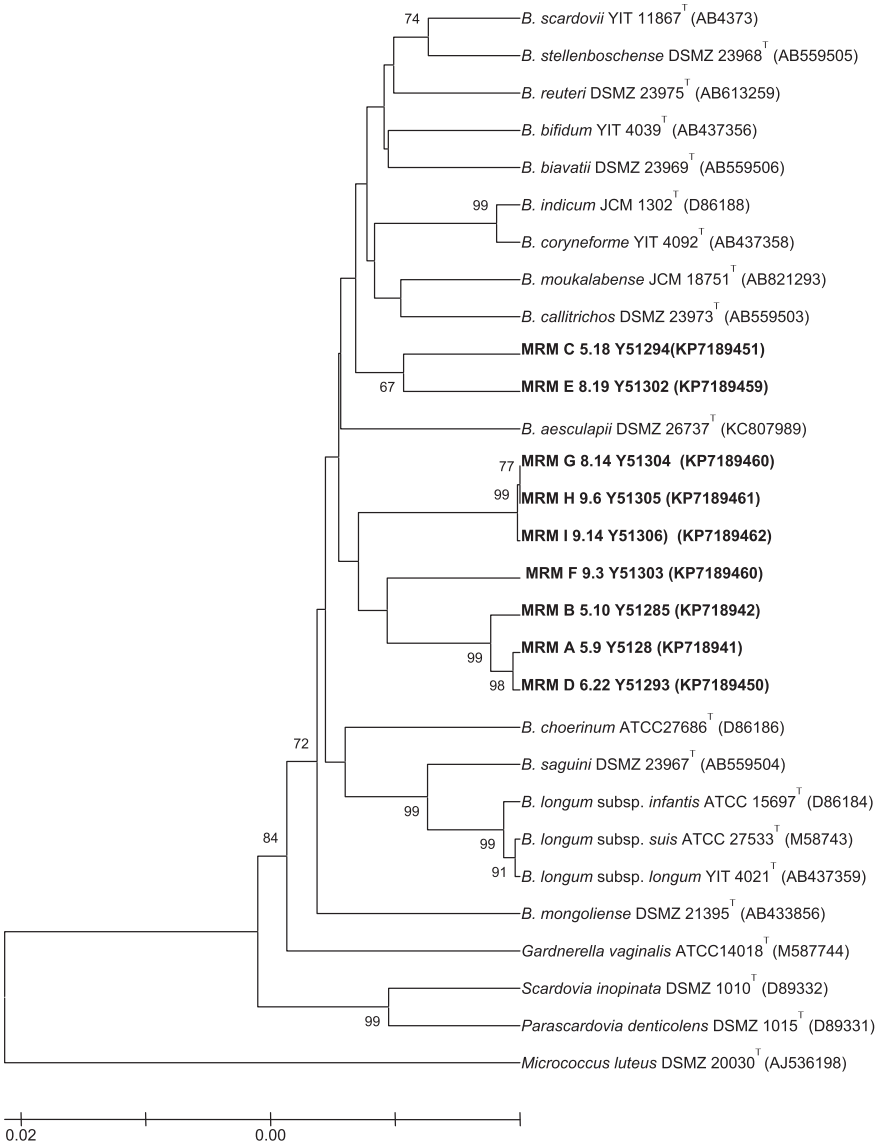

Fig. 2. Phylogenetic relationship between representatives strains from the different groups (from MRM_A to MRM_F) and representatives of related species of the genus *Bifidobacterium* based on 16S rRNA gene sequences. Evolutionary history of representative strains inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.68019710 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and *Micrococcus luteus* DSMZ 20030^T (AJ536198) was used as outgroup.

to have no cutting sites for *HaeIII* and no fragment was generated in their RFLP profiles. Strain MRM 9.3 showed too many fragments, their sum exceeding the length of the *hsp60* partial gene sequence (590 bp), whereas the profile obtained from strain MRM 6.22 appeared as a result of over digestion. Table 2 shows the RFLP-PCR profiles obtained by Stenico et al. [11], along with those obtained in this study. The percentage of occurrence of the most represented species was 30.4%, 28.2% and 13% for *B. aesculapii*, *B. callithricos* and *B. reuteri*, respectively: these species in the common marmoset have already been described [5,6].

Representative strains belonging to groups which possess RFLP profile not assigned to any bifidobacterial species (Table 2), when compared by means of sequence analysis of the 16S rRNA partial gene (ranging between 1391 and 1509 bp) on the BLAST database (www.blast.ncbi.nim.nih.gov), show a below-96% similarity to any currently known bifidobacterial species.

The 16S rRNA gene sequences of representative strains, their closest relatives and related genera were aligned using the ClustalΩ in CLC Sequence Viewer, v. 7.5 (www.clcbio.com). The phylogenetic tree (Fig. 2) was constructed with UPGMA, the Unweighted Pair Group Method with Arithmetic Mean; the Kimura 2-parameter method was employed to calculate the evolutionary distances; and the statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates in MEGA 6 [13].

The isolates MRM 5.9, MRM 5.10 and MRM 6.22 (representatives of RFLP profile groups MRM_A, MRM_B, and MRM_D, respectively), showing high similarity values (about 99.8–100%) between each other (even if they possess different RFLP profiles), can be ascribed to a single species; the same it is true for the isolates MRM 8.14, MRM 9.6 and MRM 9.14 (representatives of profile groups MRM_G, MRM_H and MRM_I, respectively) (Fig. 2). On the other hand the isolates MRM 5.18 (group MRM_C), MRM 8.19 (group MRM_E) and MRM 9.3 (group MRM_F) do not show high level of similarity between each other and between any other groups described above and for this reason they could represent 3 different species (Fig. 2).

A search for close relatives in the BLAST database, based on the 16S rRNA sequences, has been described in Table 1S. The highest similarity for groups MRM_A, MRM_B and MRM_D with *B. callithricos* (96%, 94% and 95%, respectively), *B. reuteri* (95% for MRM_A), *Bifidobacterium kashiwanohense* (94%for MRM_B and MRM_D) has been showed. MRM_G showed the highest similarity with *B. stellenboschense* and *Bifidobacterium bifidum* (94%). MRM_H and the MRM_I BLAST results gave the highest homologies with *B. bifidum* (94%), *Bifidobacterium breve* and *Bifidobacterium scardovi* (93% and 94%, respectively). MRM_C showed similarity with *B. bifidum* (96%) and *Bifidobacterium longum* subsp. *longum* (95%). MRM_E was closely related to *B. bifidum* and *Bifidobacterium coryneforme* (95%), while MRM_F with *B. stellenboschense* (95%) and *B. bifidum* (94%).

The present study represents a first description of bifidobacterial occurrence in faecal samples from babies of the common marmoset. The results of the PCR-RFLP analysis show the presence of strains belonging to *B. aesculapii*, *B. callithricos*, *B. reuteri* and the presence of five putative novel *Bifidobacterium* species. We found 80% of our baby subjects positive for *B. callithricos*, which gives support to the hypothesis proposed by Endo et al. [14], viz., to consider this species a predominant bifidobacterial species in marmoset faeces. Moreover Endo et al. [6] described the new species *B. callithricos* and *B. reuteri* from the common marmoset but described only one strain for each species. Nevertheless, the description of a new species using only one strain can have strong taxonomical relevance since, as in the present study, subsequent research in the same ecological niche could bring to light different strains of the same species in different geographical places. The marked presence in baby marmosets of *B. aesculapii*, never found in adult marmoset, could suggest a predilection of the species for the gut habitat of baby individuals, a situation similar to humans where some bifidobacterial species are peculiar only to infants [1]. Unlike other studies concerning bifidobacteria in non-human primates, no bifidobacterial human species have been found in baby common marmosets [2,3,6]. Finally, further studies are needed to establish the status of the new species for the new bifidobacterial groups from baby marmoset, [MRM_A, MRM_B and MRM_D], [MRM_G, MRM_H and MRM_I], [MRM_C], [MRM_E] and [MRM_F], as suggested in the present work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2015.03.001.

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DRAFT 2

Michelini, S., Modesto, Biavati, B. & Mattarelli, P. Cultivable *Bifidobacterium* spp. isolated and identified from faeces of cotton-top tamarin (*Saguinus oedipus*) and emperor tamarin (*Saguinus imperator*).

Bifidobiota in the faeces of the cotton-top tamarin (*Saguinus oedipus*) and the emperor tamarin (*Saguinus imperator*)

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Highlights:

- For the first time bifidobacteria have been quantified and isolated from both emperor and cotton-top tamarin
- Cotton-top tamarin and emperor tamarin gut microbiota host wide biodiversity of bifidobacteria
- Tamarins host a complex bifidobacterial community
- Non-human primates host their own bifidobacterial species
- Novel bifidobacterial species have been found exploring *Prosimians*.

ABSTRACT

Bifidobacteria represent one of the main bacterial groups of the human and animal gastrointestinal tract (GIT). Bifidobacterial species have been found to be strictly host specific, indeed while some species have been thought to be exclusively of human origin, others are exclusively associated to non-human host.

Interestingly, the species distribution of bifidobacteria in human gut was found to be different in adults when compared to infants.

However, unlike in humans, the diversity of bifidobacteria in the gut of non-human primates is poorly understood. Recent studies exploring diversity of Bifidobacteriumin monkeys, such as the common marmoset (Callithrix jacchus L.), the red-handed tamarin (Saguinus midas L.), the ring-tailed lemur (Lemur catta), and the wild gorilla (Gorilla gorilla gorilla), found 11 novel bifidobacterial species. In the present work, for the first time, the presence and distribution of cultivable bifidobacteria in the faeces of two species of tamarins, the cotton-top tamarin (Saguinus oedipus) and the emperor tamarin (Saguinus imperator), have been explored.

High levels of viable bifidobacteria were found in both subjects (>8 log10/g of faeces) and sixty-three fructose-6-phosphate phosphoketolase positive strains were isolated and characterized. Rep PCR analysis with primer BOXA1R clustered these isolates in seventeen different groups, suggesting a high intra- and inter- subjects biodiversity. The 16S rRNA gene sequence analysis revealed the presence of both 14 putative novel bifidobacterial taxa and three species previously described in Callitrichidae: Bifidobacteriumcallithricos, Bifidobacteriumtissieri, and Bifidobacteriummyosotis. Notably, four taxa were found common and shared between the two subjects. As bifidobacteria have been found in high numbers and with ten different bifidobacterial

Clusters in each animal, the use of term bifidobiota for describing such heterogeneous bifidobacterial community is now proposed. Furthermore, the recovery of species previously isolated from close related host species, such as the common marmoset, may support the hypothesis that they are more functionally interchangeable than the community of distant related host species.

Key words: *Bifidobacterium*spp., Cotton-top tamarin, Emperor tamarin, gut microbiota, novel species

Genbank accession numbers for 16S rRNA gene of strains TRE_1, TRE_2, TRE_3, TRE_7, TRE_9, TRE_15, TRE_22, TRE_24, TRE_28, TRE_33, TRE_34, TRE_B, TRE_D, TRE_E, TRE_F, TRE_H, TRE_N and TRE_Q from cotton top tamarin are KU254121, KU254122, KU291307, KU254123, KU291308, KU291309, KU291310, KU291311, KU291312, KU291313, KU254124, KU254125, KU254126, KU291314, KU254127, KU254128, KU254129 and KU291315, respectively. Genbank accession numbers for 16S rRNA gene of strains TRI_3, TRI_5, TRI_6, TRI_7, TRI_9, TRI_11, TRI_13, TRI_15, TRI_16, TRI_19, TRI_20, TRI_21, TRI_22, TRI_23, TRI_24, TRI_25, TRI_27 and TRI_28 from emperor tamarin are KU298953, KU298954, KU298955, KU298956, KU298957, KU298948, KU298949, KU298958, KU298959, KU298960, KU298950, KU298961, KU298951, KU298952, KU298962, KU298963, KU298964 and KU298965, respectively.

INTRODUCTION

The commensal microbiota in the gastrointestinal tract (GIT) is a complex community of many different species of microrganisms (bacteria, virus, yeasts and protozoa) playing an important role in host health and particularly, in nutritional, physiological and immunological functions [36]. Furthermore, diet composition, age, diseases and stressful factors may all affect the composition and the activity of both human and animal gut microbiota [9,32,45], reflecting the coevolution of the microorganisms with their animal host and the diet of the host [46]. As a result, bacterial populations can differ between individuals.

Therefore, it is important to study the intestinal microbial diversity to better understand the relationship between bacterial communities and their hosts, as well as to determine the relationship between the microbial community structure and function [16].

To date, only a few reported studies on faecal microbiota of non-human primates are available, and these have mainly focused on great Apes, such as the baboon, gorilla, [3] orangutan and chimpanzee [16,25,48]. Interestingly, a recent study on gut microbiome assembly of three species (Varecia variegata, Lemur catta, and Propithecus coquereli) in the primate clade of Lemuriformes, [19] showed differences in the distributions of their dominant phyla respect to other non-human primates. Specifically, lemurs appear to harbour ratios of Proteobacteria and Firmicutes more similar to Pan species than to either Gorilla species [25] or to Nycticebus pygmaeus (pygmy loris, the only other prosimian whose gut microbiota has been studied to date [47]) while Bacteroidetes shows the opposite relationship. Furthermore, lemur gut microbiomes contained two bacterial lineages associated with humans consuming a distinctly non-Western diet, probably due to the higher prevalence of plants and produce in non-Western diets, thus yielding the similarity to those species found in lemurs [19].

Bifidobacteria represent one of the most important bacterial groups within the *Actinobacteria,* usually present in GIT of humans as well as in a wide variety of other animals (e.g. birds, ungulates, lagomorphs and rodents) [2]. They are generally considered host-species-specific microorganisms with a quite variable occurrence and species composition in different animals, then suggesting a clear separation in “human” and “non-human” groups [22,44]. In human infants, members of the genus *Bifidobacterium*dominate the indigenous gut microbiota (until 90%) while in adults cover about 4% [39]. Bifidobacteria are often associated with health-promoting effects [27,41]

such as maintaining appropriate balance of the gut microbiota, reducing the risk of pathogen infection and modulating immune system [5].

Several studies demonstrated the importance to isolate and to identify novel *Bifidobacterium*strains from various animals including humans to better understand how they are distributed [10,38].

Ushidas et al. successfully isolated *Bifidobacterium*angulatum -like bacteria from wild chimpanzees in Bossou, Guinea [42] even though in a previous study based on sequence analyses of bifidobacterial 16S rRNA genes retrieved from faeces of chimpanzees in Mahale, Tanzania [38], wild chimpanzees had been suggested to possess non-human-type bifidobacteria. Unlike Chimpanzees in Bossou, which live close to villages with populations of about 3000 and dense agricultural fields, chimpanzees in Mahale live in remote areas far from human agricultural activity. In preliminary experiments, a partial bifidobacterial 16S rRNA gene similar to those of the chimpanzees in Mahale was retrieved from the faeces of a wild lowland gorilla (Gorilla gorilla gorilla) in Gabon. Thus, the presence of species of the genus *Bifidobacterium*associated with great Apes was suggested and in a subsequent study a novel species, *Bifidobacterium*moukalabense was finally described [38].

We also isolated human-type bifidobacteria, viz. *Bifidobacterium*dentium and *Bifidobacterium*adolescentis from faeces of chimpanzees (Pan troglodytes) and orangutan (Pongo pygmaeus) respectively, housed at Borås Djurpark, a zoo in the northern part of central Borås [8].

However, little is known about the bifidobacterial species distribution among ancestral primates, such as lemurs and New World monkeys. Endo et al. (2012) recently described two new species of bifidobacteria from the faeces of a common marmoset (Callithrix jacchus L.) viz. *Bifidobacterium*reuteri and *Bifidobacterium*callitrichos, and three new species from the faeces of a red-handed tamarin (Saguinus midas L.) viz, *Bifidobacterium*biavatii, *Bifidobacterium*stellenboschense and *Bifidobacterium*saguini. Studying the bifidobacterial distribution in non-human primates we successfully isolated four novel species and two putative novel taxa from the faeces of five baby subjects of the common marmoset, viz. *Bifidobacterium*aesculapii, *Bifidobacterium*myosotis, *Bifidobacterium*tissieri and *Bifidobacterium*hapali, strain MRM 8.19 and strain MRM 9.3 currently under investigation, [22,23], and two novel species from the faeces of an adult subject of the ring tailed lemur (Lemur catta) [24] and of an adult subject of the black lemur (Eulemur macaco) [21] viz. *Bifidobacterium*lemurum and *Bifidobacterium*eulemuris, respectively.

For this study we explored the presence and the diversity of cultivable bifidobacteria in the faecal samples of two species of tamarins, the cotton-top tamarin (Saguinus oedipus) and the emperor tamarin (Saguinus imperator) housed under semi natural conditions in Parco Natura Viva, Pastrengo, Verona, Italy. Tamarins are squirrel sized New World monkeys from the family of Callitrichidae, in the genus Saguinus. Tamarins range from the southern Central America through central South America, where they are found in northwestern Columbia, the Amazon basin and the Guianas.

They are generally described as insectivore-frugivores with a diet including fruits and insects [33], always supplemented with plant exudates (gum and/or sap), nectar, reptiles and amphibians [34,37].

The main objective of this study was the isolation and the identification of bifidobacterial strains from one adult subject of the cotton top tamarin (Saguinus oedipus) and from one adult subject of the emperor tamarin (Saguinus emperor). The analysis of their bifidobacterial communities by molecular methods will be also assessed. In this context, applying both culture dependent and culture independent techniques we aimed to better characterize and examine how the faecal bifidobacterial communities are distributed and if there are differences between the two species of tamarins analysed.

MATERIAL AND METHODS

In February 2014 individual fresh faeces of two adult subjects of the cotton-top tamarin (Saguinus oedipus) and the emperor tamarin (Saguinus imperator), housed in Parco Natura Viva, (Pastrengo, Verona, Italy) were collected from the ground using a sterile spoon, put into a sterile plastic tube and stored under anaerobic conditions in an anaerobic jar (Merck) at 4 °C. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD).

Samples of fresh faeces were collected by the animal-care staff (keepers) during their routine cleaning of the enclosure, and were taken promptly to the laboratory (within 2 h). Animals were free from intestinal infections

and did not receive antibiotics or probiotics for two months before samples were collected. The diet consisted in live larvae of Tenebrio molitor or, alternatively, Zophobas moiro, fresh fruit and gum Arabic. In addition, twice per week the diet is supplemented with fresh vegetables, cocked vegetables like peas, hard boiled eggs, coocked meat like turkey, rice, baby cereal food. Primates jellies, seeds, nuts, yogurt, fresh chees, dry fruits, honey are used as food enrichment. Moreover a mineral-vitamin supplement for primates is added twice per week to the baby food [28].

Isolation and enumeration of *Bifidobacterium*spp.

For bifidobacteria isolation and enumeration, faecal samples of the material (approx. 1–2 g) from each animal were serially diluted (ten fold) with Peptone Water (Merck) supplemented with cysteine hydrochloride (0.5 g/L); aliquots of 1 ml from each dilution (from 10-1 down to 10-9) were inoculated onto TPY agar [31] supplemented with mupirocin (100mg/L) (Applichem). Plates were incubated in anaerobic conditions, at 37°C for 48-72 hours. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD). After incubation, morphologically different colonies were randomly picked-up and re-streaked for several generation in order to isolate purified individual bacterial strains. Isolated pure strains were suspended in a 10% (w/v) sterile skim milk solution, supplied with lactose (30 %) and yeast extract (0.3 %) and kept both freeze dried and frozen at –120°C until further analysis.

Identification of bacterial isolates

The selected isolates were observed by optical microscopy to determine their morphology and Gram staining results. Additionally, they were tested for catalase and oxidase activities, and for motility. Gram staining, catalase and oxidase activities were assessed using cells grown on TPY agar at 37 °C for 48 h under anaerobic conditions using individual Gram-staining reagents (Merck Millipore), a 3% (v/v) hydrogen peroxide solution and cotton swabs impregnated with N, N, N′, N′-tetramethyl p-phenylenediamine dihydrochloride and dried (Oxibioswab; Biolife), respectively. Strain motility was determined by stabbing the culture into TPY medium containing 0.4% agar, knowing that motile strains show a diffused growth spreading away from the line of inoculation.
All the gram-positive and catalase/oxidase-negative isolates with typical bifidobacterial shapes were identified to the genus level by evidence of fructose-6-phosphate phosphoketolase (F6PPK) activity in cellular extracts. Detection of F6PPK activity was carried out according to the method described by Scardovi [31] and modified by Orban & Patterson [26].

Genomic DNA extraction

For REP-PCR and 16S rRNA genes sequence analysis, the genomic DNA of each strain was extracted by using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instruction with slight modifications [24]. Briefly, pelletted cells from overnight cultures were washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), re-suspended in TE containing 50 mg lysozyme ml-1 and incubated in a water bath at 37°C for 80 minutes. After adding 600 µl of Nucleic Lysis Solution to the lysate, an incubation step of 15 minutes at 80°C was also carried out. All subsequent manipulations were performed according to manufacturer's instruction. The DNA concentration was determined spectrophotometrically from the A_{260} and the purity of each sample was estimated by determining the A_{260}/A_{280} ratio.

BOX PCR Analysis

For discrimination of the isolates, BOX-PCR fingerprinting was carried out using the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') [17]. The 20 µl of final reaction mixture volume contained 1.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each dntps, deoxynucleoside triphosphate, (HotStartTaq plus DNA polymerase MasterMix kit, Qiagen), additional 0.05 mM of dNTPs, 70 ng of DNA template and 2 µM of each primer. The PCR amplification was performed in an Applied Biosystem Verity Thermal cycler (Applied

Biosystems, Foster City, CA) with the following temperature profile: initial denaturation step at 94°C for 3 min, 30 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min an extension at 65°C for 8 min, and a final extensions step at 65°C for 16 min. Amplicons (20 µl) were fractioned by electrophoresis on a 2% agarose (w/v) gel at a voltage of 7 V/cm. Gels were ethidium bromide stained (0.5 µn/ml) and the fingerprinting profiles visualized under 260 nm UV light (Molecular Imager Gel Doc XR (BIO-Rad). The banding patterns from BOX-PCR were first acquired by Image lab software (BioRad) and subsequently analysed with Gel Compare II software version 6.6.11 (Applied-Maths, Ghent, Belgium). The similarities between strains were calculated using the Jaccard similarity index with optimization and tolerances set to 4 and 1.5%, and the dendrograms were obtained by means of the Unweighted Pair Group Method with Arithmetic Average (UPMGA) clustering algorithm with correlation levels expressed as percentage values of the Jaccard similarity index (Fig. 1-2).

16S rRNA genes sequence analysis

The 16S rRNA partial gene sequences were amplified from all isolated strains using the primer pair Bif 285 5'-GAGGGTTCGATTCTGGCTCAG-3' and 261 5'-AAGGAGGTGATCCAGCCGCA-3') [14]. The reaction was performed in 20 µl of PCR mixtures containing 1.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each deoxynucleoside triphosphate (HotStartTaq plus DNA polymerase MasterMix Kit; Qiagen), 0.1 µM of each primer and 25 ng of DNA template for 16S rRNA. Amplifications were carried out in a TGradient thermal cycler (Biometra). The 16S rRNA gene was amplified with a touch down PCR performed as follows: initial denaturation (95 °C, 5 min) for HotStart Taq plus activation; 4 cycles with denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s, and extension at 72 °C for 90 s; 21 cycles with denaturation at 94 °C for 60 s, annealing at 60°C for 60 s, and extension at 72 °C for 90 s; 15 cycles with denaturation at 94 °C for 60 s, annealing at 58°C for 60 s, and extension at 72 °C for 90 s; the PCR was completed with a single elongation step (10 min at 72 °C). Resulting amplicons were separated on 2 % agarose gels, followed by ethidium bromide staining and PCR fragments were purified using the NucleoSpin Gel and PCR clean up kit (Macherey-Nagel, Duren, Germany) following manufacturer's instructions.
PCR products were purified with NucleoSpin (Macherey-Nagel GmbH & Co. KG, Germany) following the manufacturer's instructions. All purified amplicons were directly sequenced by Eurofins MWG Operon Biotech (Germany).
About 800 bp of the amplified 16S rRNA gene were sequenced from all the isolates and used for BLAST search against GenBank (http://blast.ncbi.nlm.nih.gov) whereas the pairwise nucleotide sequence similarity values were calculated using LAlign server (http://embnet.vital-it.ch/software/LALIGN_form.html) which provides a web-based tool.
The 16S rRNA gene sequence (about 1350 bp) assembly was performed only on sequences from the representative strains of BOX PCR clusters, using CLC Sequence Viewer version 7.5 for Mac OS (CLC, Inc., Aarhus, Denmark). After editing, the closest known relatives of the novel strains were determined by comparison with database entries, and sequences of members of closely related species were retrieved from the EMBL and GenBank nucleotide databases. Pairwise nucleotide sequence similarity values were calculated using the LAlign program (http://embnet.vital-it.ch/software/LALIGN_form.html).
A phylogenetic tree based on a total of 38 partial 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium*isolated from non human primates and their related neighbors, was reconstructed with the maximum-likelihood [6] method and the evolutionary distances were computed by the Kimura 2-parameter method [15] using mega version 6.0 [35]. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates [12] and Micrococcus luteus DSM 20030T (Figg. 3-6) was used as an outgroup.

Real time PCR quantification of bifidobacteria

Quantification of *Bifidobacterium*spp. in the faeces of both tamarins was also carried out with real-time PCR.
The genomic bacterial DNA was extracted from faeces using the QIAamp Fast DNA Stool Mini Kit (Qiagen) following the manufacture's instruction but modifying the lysis step. Briefly, about 0.200 g of faecal samples

were homogenized in 2ml eppendorf with 650 µl of InhibitEX buffer from the kit and 0.5 g of sterile glass beads (0.1 mm in diameter) by vortexing. The suspension was incubated at 95°C for 15 minutes and cooled on ice for 2 minutes. The supernatant was recovered by centrifugation at 15.000 rpm for 2 minutes, and transferred to a new 2 ml tube. To improve the DNA extraction, the procedure was repeated adding 650 µl of InhibitEX buffer to the pellet. The recovered supernatant was joined to the previous one and the manufacture's protocol was followed to complete the extraction. Extracted DNA was stored at −20 °C. Purity and concentration of DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200PRO NanoQuant, Tecan, Mannedorf, Switzerland).

Amplifications were performed with the StepOne Real-Time PCR system (Thermo Fisher Scientific) using the primer pair xfp-fw (5′-ATCTTCGGACCBGAYGAGAC-3′) and xfp-rv (5′-CGATVACGTGVACGAAGGAC -3′), targeting a 235 bp region of the xfp gene [7]. The 20 µl of amplification mixture containing 10 µl of SYBR Select® Master Mix (Applied Biosystems), 0.4 µM of each primer and 20 ng of DNA. The amplification conditions consisted of an initial cycle of 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s and 62,5°C for 1 min. The qPCR reactions were performed in MicroAmp optical plates sealed with optical adhesive covers (Applied Biosystems, Foster City, CA, USA). Thermal cycling, fluorescent data collection and data analysis were carried out with StepOne sequence detection system (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's instructions.

Melting curve analyses were performed by slowly increasing the temperature from 60 °C to 95 °C. Measurements were performed in triplicate, and were repeated when variation between measurements exceeded 0.5 Ct.. Data obtained from amplificaton were transformed to obtain the number of bacterial cells per gram of faeces, expressed as log colony forming unit (CFU)/g. Standard curves were made by plotting cycle threshold (CT) values, against dilutions of the quantitative standard (xfp PCR fragment) for which the number of gene copies was known. For bifidobacteria, which harbour a single copy of xfp gene per cell, the measured CT value was directly proportional to log xfp gene copy number and consequently to log10 cell number. The amplification efficiency was calculated from the slope of the standard curve using the formula: $E=(10^{-1/slope}/2)\cdot 100$.

For bifidobacterial quantification, a PCR fragment of the xfp gene was used as an internal standard. Therefore, DNA was extracted from a pure culture of *Bifidobacterium*longum subsp. longum ATCC 15708, as above described. Amplification of this DNA was performed with primers xfp-fw and xfp-rv in a Biometra Gradient PCR apparatus (Biometra, Gottingen, Germany) in a 20 µl of PCR mixtures containing 1.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each deoxynucleoside triphosphate (HotStartTaq plus DNA polymerase MasterMix Kit; Qiagen), 0.1 µM of each primer and 25 ng of DNA template. The conditions for PCR consisted of an initial cycle of 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 1 min, and a final polymerization step of 10 min at 72°C. The PCR product (235 bp) was then purified using the NucleoSpin (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions and photometrically quantified. Different dilutions of purified PCR product (128 ng, 12.8 ng, 1.28 ng, 128 pg, 12.8 pg, 1.28 pg, 128 fg, 12.8 fg, 1.28 pg, 0,128 pg DNA) were used as a template for the standard curve. One nanogram of *xfp* PCR product corresponded to $4.2 \cdot 10^9$ *xfp* copies. The qPCR assays were replicated three times independently.

RESULTS AND DISCUSSION

Isolation, enumeration and identification of isolates

The microbial ecology of the intestinal tract involves a large number of bacteria and represents a complex community that reflects the coevolution of the microorganisms with their animal host and the diet of the host [47]. Furthermore, the composition of this microbiota varies among individuals of a given species as well as within individuals across time, due to a number of factors such as diet, age and sex. Therefore, studying the intestinal bacterial diversity is important for better understanding the relationship between bacterial communities and their hosts, and determining the relationship between the microbial community structure and function.

Previous studies have proven that the microbiomes of non-human primates exhibit a much higher similarity with those of primates than with other animals. Therefore, the study of the microbiota from these non-human primates could provide important insights into the reflection of their features in humans [17].

An approach integrating culture-based and molecular methods was applied here in order to obtain a detailed assessment of the bifidobacterial diversity and distribution in the two species of tamarin, cotton top tamarin (Saguinus oedipus) and emperor tamarin (Saguinus imperator). Culture-based analyses were aimed at a quantitative assessment of the microbial loads of bifidobacteria, and at the isolation and characterisation of cultivable strains.

The bifidobacteria gene copy numbers (CN) were assessed in both stool samples by qPCR targeting the xylulose-5-phosphate/fructose-6-phosphatephosphoketolase gene (xfp) gene. The primates included in the present study resulted positive for bifidobacteria and interestingly the mean bifidobacteria CN in the cotton top tamarin was log 9,63 SD +/− 0.03 copies per gram faeces whereas in the emperor tamarin it resulted log 9,13 SD +/− 0.01 copies per gram faeces.

Bifidobacterial counts were also determined in both the subjects using a mupirocin-based medium (mTPY), which has been previously described to be selective for bifidobacteria [29]. Results confirmed an abundance of bifidobacteria in both the subjects, with high numbers of colony counts ranging from 8.0 to 9.0 log10 CFU/g.

To analyse the biodiversity of the culturable bifidobacterial population between two faecal samples, morphologically different colonies, assumed to represent the dominant flora of a given sample, were randomly picked-up from plates obtained by serial dilutions, and a total of 63 bacterial isolates (45 from the cotton top tamarin and 19 from the emperor tamarin) were thus collected and at first characterized by means of phenotypical tests. All isolates resulted Gram-positive staining, non-motile, catalase and oxidase negative, and showed F6PPK activity.

Chromosomal DNA was extracted from all strains and used for REP-PCR analyses. The hierarchical numerical analysis of the generated BOX banding patterns is shown in three dendrograms (Supplementary Fig. 1-3). BOX PCR failed to find a clear strain clustering. Indeed, as determined by UPGMA clustering algorithm, the sixty-three isolates groped in more than 10 different clusters for each animal. It has been reported that the current BOX-PCR technique, in which the amplified products are separated by agarose gel electrophoresis, suffers from several limitations like poor band resolution and run standardization for comparison of the different profiles in different gels [4]. To overcome these limitations comparative analysis of the 16SrRNA gene sequences was used to obtain a very well defined grouping.

The 16S rRNA gene sequence was amplified and partially sequenced (about 800 bp) from almost all strains in each animal. Comparative analysis of the 16S rRNA partial sequences identified 17 different independent clusters of which 4 (Cluster I, III, V and VII) were common and shared between the 2 animals (Table 1 and 2). Furthermore, representative strains for each Cluster were selected and identified at species level by means of almost complete 16S rRNA gene (about 1200 bp) sequencing analysis. All obtained sequences were submitted to GenBank.

Table 1. Resulting clusters of strains isolated from the cotton-top tamarin. Grouping derives from the match of cluster analysis and 16S gene sequence comparison. In parenthesis the number of strains is reported. The 16S rRNA sequences were amplified and sequenced from strains indicated in bold.

Cluster I (4)	**TRE_7, TRE_17, TRE_26 TRE_33,**
Cluster II (1)	**TRE_C**
Cluster III (1)	**TRE_M**
Cluster IV (19)	**TRE_1, TRE_3**, TRE_4, TRE_5, TRE_6, **TRE_9**, TRE_10, TRE_11, TRE_12, TRE_14, **TRE_15**, TRE_16, TRE_21, TRE_27, TRE_29, TRE_30, TRE_31, TRE_A, TRE_G
Cluster V (6) **(Identified as *B.callithricos*)**	**TRE_2**, TRE_8, TRE_13, **TRE_28**, **TRE_B**, **TRE_Q**
Cluster VI (1)	**TRE_H**

Cluster VII (6)	TRE_19, TRE_20, **TRE_22**, TRE_23, **TRE_24**, **TRE_34**
Cluster VIII (4)	**TRE_E,** TRE_O, **TRE_N**, TRE_L
Cluster IX (1)	**TRE_D**
Cluster X (1) **(Identified as *B. myosotis*)**	**TRE_F**

Table 2. Resulting clusters of strains isolated from the emperor tamarin. In parenthesis the number of strains is reported. The 16S rRNA sequences were amplified and sequenced from strains indicated in bold.

Cluster I (1)	**TRI_24**
Cluster III (3)	**TRI_3, TRI_22, TRI_27**
Cluster V (1) **(Identified as *B.callithricos*)**	**TRI_16**
Cluster VII	**TRI_9, TRI_11**
Cluster XI (5)	**TRI_5,** TRI_17**, TRI_20**, **TRI_21**, **TRI_23**
Cluster XII (1)	**TRI_6**
Cluster XIII (1)	**TRI_7**
Cluster XIV (1)	**TRI_13**
Cluster XV (2) **(Identified as *B. tissieri*)**	**TRI_15, TRI_25**
Cluster XVI (1)	**TRI_19**
Cluster XVII (1)	**TRI_28**

Each 16S rRNA gene sequence generated from each reference strain originating from the cotton top tamarin and from the emperor tamarin was subjected to a Blast search against GenBank and to pairwise sequence similarity calculation with LAlign. Comparative sequence analysis of the 16S rRNA gene is commonly used to determine the phylogenetic position of novel isolates. Strains that show over 97% of 16S rRNA sequence identity are considered to belong to the same species [18,43]. All the 16S rRNA gene sequences obtained from strains assigned to Cluster V, Cluster X and Cluster XV showed the highest similarity values to *Bifidobacteriumcallithricos* DSM 23973T (99.1%) to *Bifidobacteriummyosotis* DSM 100196T (99.1%) and to *Bifidobacteriumtissieri* DSM 100201T (99.0%) respectively, as obtained with LAlign (Table 3-4). Interestingly, *B. callithricos*, corresponding to Clusters V, was shared across the two different species of tamarin whereas *B. myosotis*, corresponding to Cluster X and *B. tissieri*, corresponding to Cluster XV have been found only in Saguinus oedipus and in Saguinus imperator, respectively.

Cluster VII, VIII and XII showed 16S rRNA similarity values over 97% to *B. tissieri* (97.6%), *B. stellenboshense* (97.7 %) and *B. biavatii* (97.7%), respectively. Cluster XVI showed a similarity value of 97.6% with strain MRM 8.19, which was firstly isolated from the baby common marmoset and is currently under investigation to be proposed as new taxon [20]; at last Cluster XIV showed a similarity value of 97.4% to Cluster XVI. The similarity values of Clusters VII, VIII, XII, XIV and XVI are near the cut-off values for definition of new taxa (97%) and for this reason further studies are needed to define their taxonomic status.

All 16S rRNA gene sequences from other Clusters (I, II, III, IV, VI, IX, XI, XIII, XVII) showed a similarity above 97% to each other and to their nearest database entries, and thus nine novel putative bifidobacterial taxa, were retrieved: 4 in cotton top tamarin, 3 in emperor tamarin and 2, corresponding to Clusters I and III, shared by two tamarin species.

Table 3. 16S rRNA partial gene similarities between each cluster retrieved in the cotton top tamarin and their related species obtained using the LAlign tool.

Species	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI	Cluster VII	Cluster VIII	Cluster IX	Cluster X
Cluster I		94.5	94.6	93.7	94.2	93.1	94.8	95	93.9	94
Cluster II	94.5		95.6	94.1	94.3	94	95.5	93.4	93.9	93.5
Cluster III	94.6	95.6		93.9	94.9	94.5	94.8	93.8	94.2	94.4
Cluster IV	93.7	94.1	93.9		94.2	93.0	96.7	93.7	92.4	93.4
Cluster V	94.2	94.3	94.9	94.2		94.9	94.4	94.2	94.6	94.7
Cluster VI	93.1	94	94.5	93.0	94.9		93.4	93.3	96.4	93.8
Cluster VII	94.8	95.5	94.8	96.7	94.4	93.4		94.7	93.3	94.9
Cluster VIII	95	93.4	93.8	93.7	94.2	93.3	94.7		92.9	94.2
Cluster IX	93.9	93.9	94.2	92.4	94.6	96.4	93.3	92.9		93.7
Cluster X	94	93.5	94.4	93.4	94.7	93.8	94.9	94.2	93.7	
MRM_8.19				95.9						
B. callithricos DSM 23969^T					99.1					
B. scardovii DSM 13734^T	96	94.9	94.1							
B. longum subsp. *longum* ATCC 15697^T						96.8				
B. tissieri DSM 100201^T							97.6			
B. stellenboschense DSM 23968^T								97.7		
B. breve DSM 20213^T									96.1	
B. myosotis DSM 100196^T										99.1

Table 4. 16S rRNA partial gene similarities between each clusters and each related species obtained using the LAlign tool.

Species	**Cluster I**	**Cluster III**	**Cluster V**	**Cluster XI**	**Cluster XII**	**Cluster XIII**	**Cluster XIV**	**Cluster XV**	**Cluster XVI**	**Cluster XVII**
Cluster I		95.2	94.2	94.1	95.7	91.7	92.7	93.9	94.3	95.8
Cluster III	95.2		95.1	95.3	95.2	91.2	93	93.6	94.5	95.1
Cluster V	94.2	95.1		94.4	94.1	91.4	94.5	93.1	94.3	95.2
Cluster XI	94.1	95.3	94.4		93.7	93.7	93.2	93.3	94.2	96.9
Cluster XII	95.7	95.2	94.1	93.7		90.5	92.4	92.5	93.5	94.7
Cluster XIII	91.7	91.2	91.4	93.7	90.5		91.9	91	91.8	94.4
Cluster XIV	92.7	93	94.5	93.2	92.4	91.9		96.4	97.4	93.2
Cluster XV	93.9	93.6	93.1	93.3	92.5	91	96.4		94.6	93.5
Cluster XVI	94.3	94.5	94.3	94.2	93.5	91.8	97.4	94.6		94
Cluster XVII	95.8	95.1	95.2	96.9	94.7	94.4	93.2	93.5	94	
B. scardovii DSM 13734^T	96	94.1								
B. callithricos DSM 23969^T			99.1							
B. breve DSM 20213^T				95.6						
B. biavatii DSM 23969^T					97.7					
B. saguini DSM 23967^T						96				96.8
B. indicum DSM 20214^T							95.2			
B. tissieri DSM 100201^T								99.0		
MRM 8.19									97.6	

The taxonomic affinities of the strains were confirmed by means of 16S rRNA gene phylogenetic tree topology. A phylogenetic tree was reconstructed based on the 16S rRNA gene sequences of the type strains of the closest neighbours and of the other bifidobacteria related to the gut of non-human primates retrieved from the GenBank database (available at http://www.ncbi.nlm.nih. gov/nuccore) (Fig. 1-3). The new bacterial isolates assigned to Clusters II, IV, VII, XIV, XV and XVI were phylogenetically related to *Bifidobacteriumtissieri* in the *Bifidobacteriumbifidum* subgroup. Strains located in Clusters VI, IX, XI, XII and XVII were related to *Bifidobacteriumreuteri* and to *Bifidobacteriumsaguini* in the *Bifidobacteriumlongum* subgroup. Finally, strains in Clusters I and III were related to *Bifidobacteriumhapali*, strains in Cluster V to *Bifidobacteriummoukalabense*, strains in Cluster VIII to *Bifidobacteriumstellenboschense* and strain in Cluster XII was related to *Bifidobacteriumscardovii* (Fig. 3).

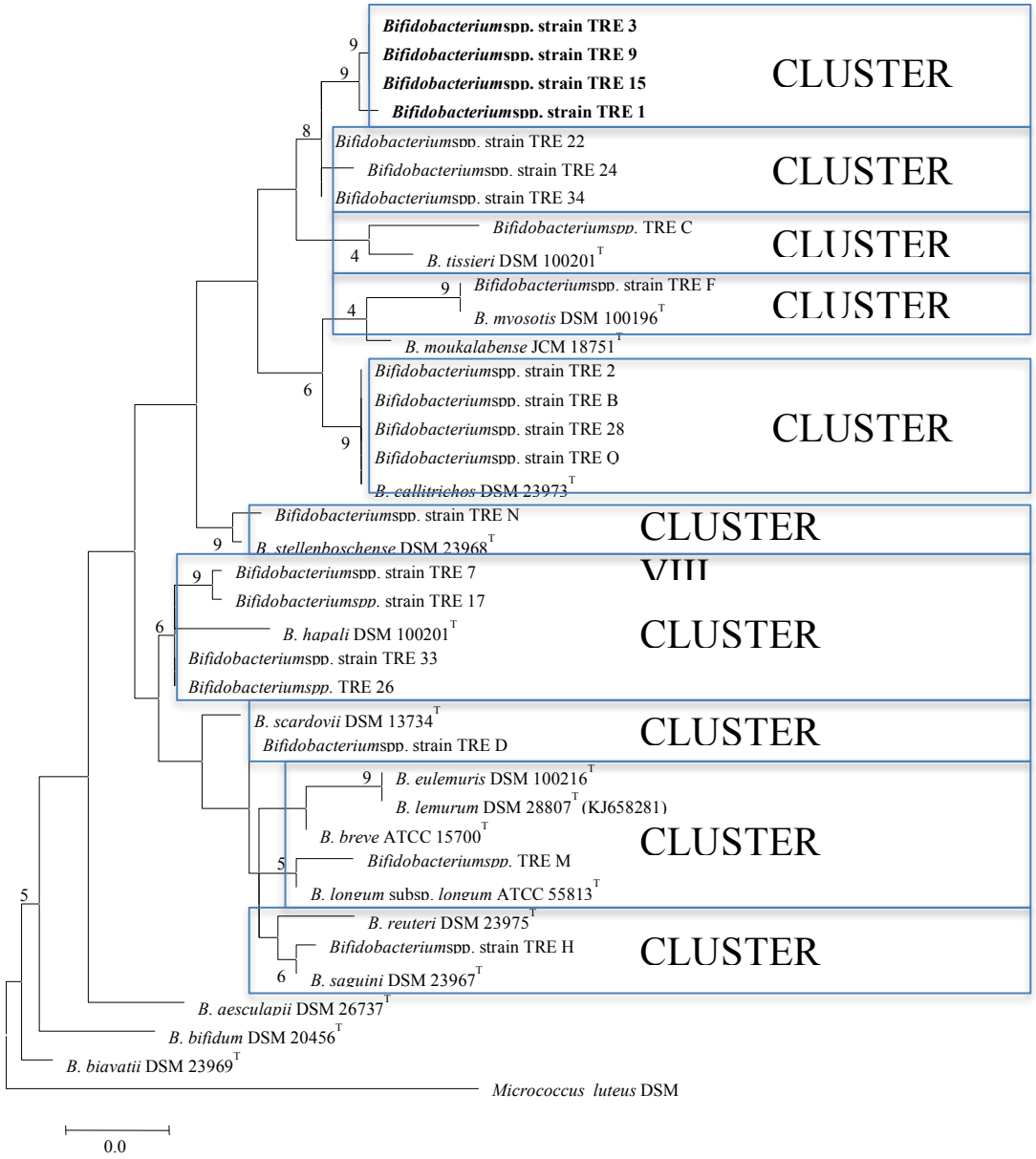

Figure 1. Phylogenetic relationship between strains from cotton-top tamarin based on 16S rRNA gene sequences. The tree was constructed by the maximum likelihood method and rooted with *Micrococcus luteus* DSM 20030^T. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap percentages above 40 are given at branching points.

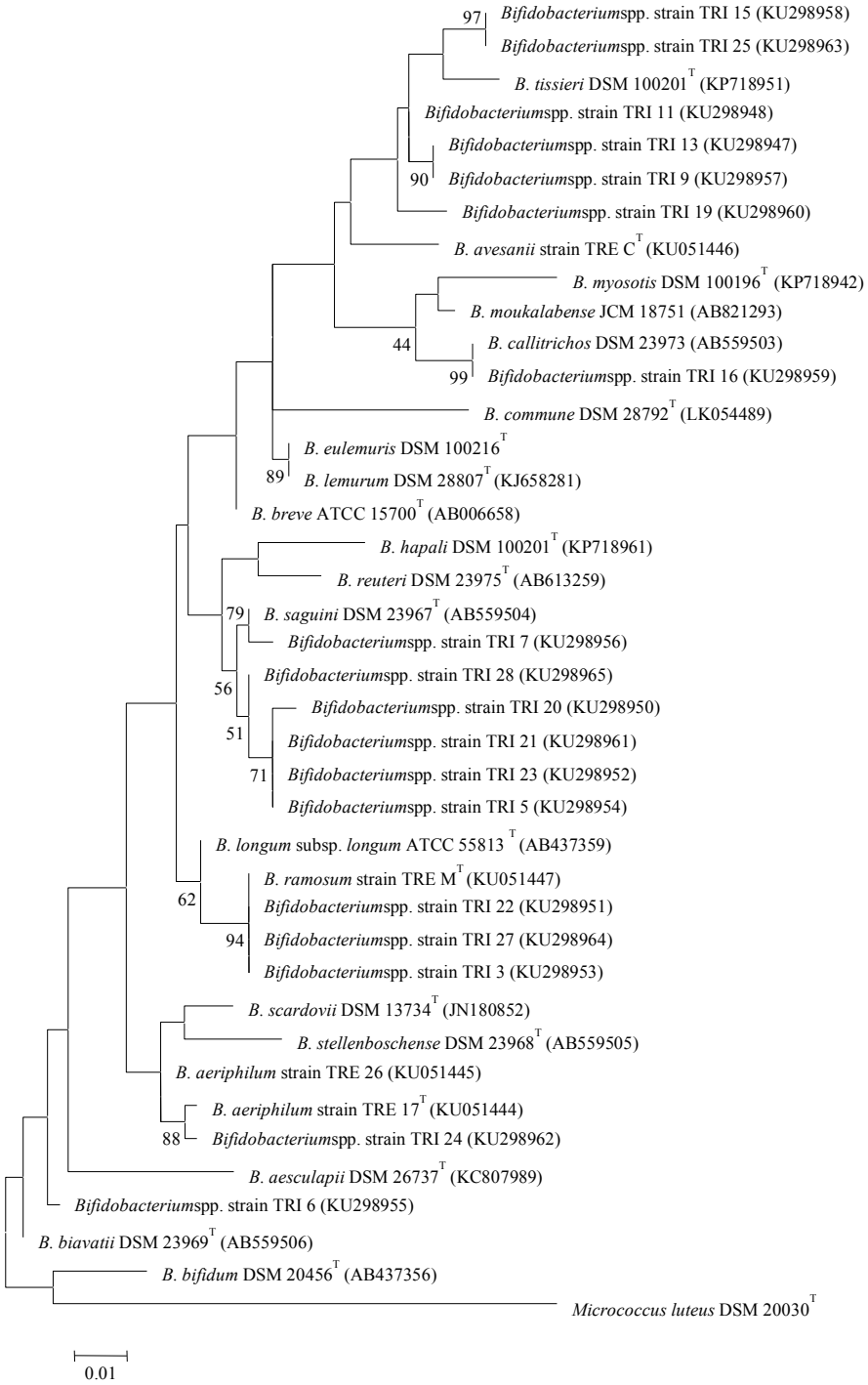

Figure 2. Phylogenetic relationship between strains from emperor tamarin based on 16S rRNA gene sequences. The tree was constructed by the maximum likehood method and rooted with *Micrococcus luteus* DSM 20030^T. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap percentages above 40 are given at branching points.

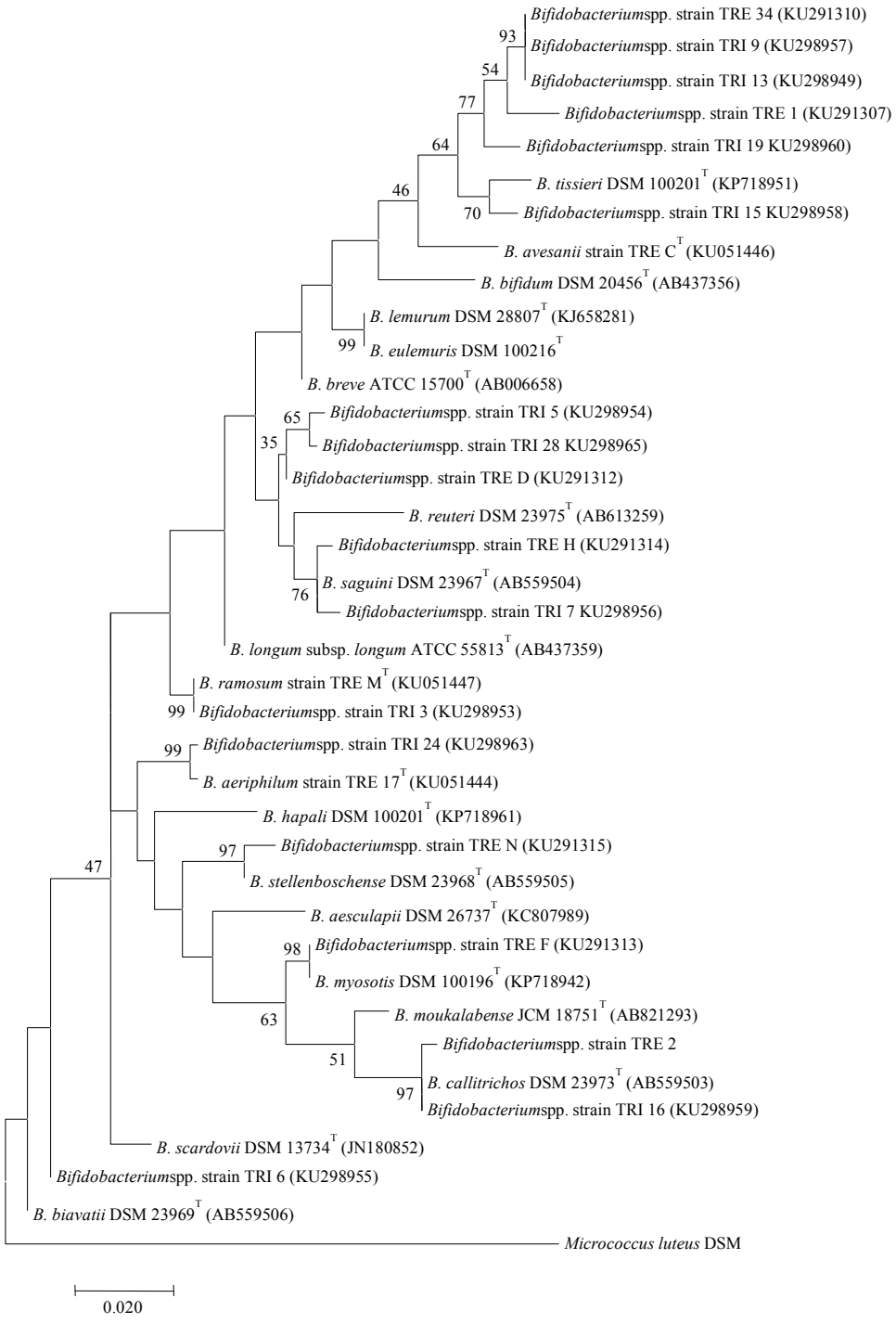

Figure 3. Phylogenetic relationship between representative strains from tamarins based on 16S rRNA gene sequences. The tree was constructed by the maximum likehood method and rooted with Micrococcus luteus DSM 20030T. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap percentages above 40 are given at branching points.

Considering our results, bifidobacteria were found as natural inhabitants of the gut of the cotton-top tamarin and gut of the emperor tamarin. Furthermore, as bifidobacteria have been found in high numbers and with ten different bifidobacterial Clusters in each animal, the use of term bifidobiota for describing such heterogeneous bifidobacterial community is now proposed.

The bifidobiota communities deriving from the faecal samples of these two tamarins revealed intra/inter-subjects diversity and similarity. Some species, such as B. callithricos, B. myosotis and B. tissieri, resulted common and shared not only between the two tamarins but also with the baby common marmosets [20,22,23], thus yielding some important considerations about non-human primate gut microbiota.

As often reported, the functional diversity of the microbial community populating the gastrointestinal tract (gut microbiota) has been shown to affect development, health, fitness and evolutionary trajectory in both humans and other animals. A number of drivers have been described to influence gut microbiota composition, including diet, physiology and evolutionary history. In addition, the physical environment may also determine interspecific and interpopulation differences [1].

In our previous studies we have described the presence of *B. callitrichos, B. reuteri* and of four different novel species (*Bifidobacteriumaesculapii, Bifidobacteriummyosotis, Bifidobacteriumtissieri*, *Bifidobacteriumhapali*) in five subjects of the Family *Callitrichidae*, viz. the baby common marmoset [20,22,23] kept in animal houses in Verona, Italy. Furthermore, Endo et al. [11] had firstly described *B. callitri*chos in a common marmoset kept in animal houses in Western Cape, South Africa. In this work we found *B. callitrichos, B. myosotis* and *B. tissieri* as common bifidobacterial taxa shared between two different species of tamarin, kept in semi-natural conditions at Parco Natura Viva, Verona, Italy. Notable, all these studied animals (viz. baby common marmosets and tamarins) were geographically separated, and, unexpectedly, in the baby common marmosets, which live in captivity and close to humans, no human-type bifidobacteria were found.

Therefore, in these different genera and species of *Callithrichidae*, the evolutionary history rather than diet or physiology seem to drive the presence of the shared bifidobacterial taxa. Indeed, as observed by De Filippo and co-workers [1] when gut microbiota diversity is observed in sympatric animals (co-occurring in the same geographical location) it is due to environmental factors, whereas when diversity is observed in allopatric animals (geographically separated) it is due to heritable factors.

Despite the accumulating data provided by modern molecular techniques, current knowledge does not yet offer a definition for a normal or optimal gut microbiota composition. In parallel with mining the entire diversity of host-associated microbial communities, recently significant effort has been devoted to a more focused approach that aims to define a core microbiota that is potentially shared across adult individuals [30].

The specific interest towards universally shared bacteria arises from the fact that, in contrast to transient gut inhabitants that fluctuate depending on the diet and other environmental factors, the common core bacteria are conserved during the mutual coevolution of host and his intestinal microbes [13,30].

As results from these considerations, we can suppose that in New World Monkeys bifidobacteria constitute an important part of the core gut microbiota even tough metagenomic studies have revealed their low abundance or even apparent absence [19,46,47]. However, the reason for a low detection of bifidobacterial species by culture-independent investigations is most likely due to technical biases, in particular those related to DNA extraction protocols and/or the PCR primers used. Therefore, caution must be applied in the interpretation of the results obtained by various published metagenomic studies of the microbial biodiversity [40]. On the other hand, only a few reported culture-independent studies on faecal microbiota of non-human primates are available, and these have mainly focused on great apes, such as the baboon, gorilla, [3] orangutans and chimpanzee [17,26,41].

Even more importantly, comparative studies of bifidobacterial distribution in New World Monkeys revealed that certain identified bifidobacterial species were exclusively present in the faeces of the common marmoset or in the tamarins, whereas other species were widely shared across *Callitrichidae*. Consequently, such complex and heterogeneous bifidobiota seems to be composed of some bifidobacterial species more intimately close to their host (probably being genetically adapted to the diet of their own host) and a core of species potentially shared across different hosts species, which reflect the co-evolution of these beneficial microorganisms with their host.

CONCLUSION

Forty-five and nineteen strains of bifidobacteria were isolated from the cotton-top tamarin and the emperor tamarin, respectively. Analysis of the BOX-PCR fingerprinting of the isolates revealed the presence of ten independent clusters in each animal, with a total of seventeen taxa. The 16S rRNA gene sequences analysis revealed in both subjects the presence of *B. callithricos*, a species that was originally described in the red-handed tamarin (*Saguinus midas*) [10]. Interestingly, the species *B. myosotis* and *B. tissieri*, originally described in the faeces of the baby common marmoset [22], were now found in the cotton-top tamarin and in the emperor tamarin, respectively.

The next future goal of this work will be the tamarin gut microbiota NGS (next generation sequencing) analysis aimed to better clarify which species constitute the core bifidobacterial community (or bifidobiota) of tamarins and which species were shared among *Callitrichidae*.

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DRAFT 3

Michelini, S., Modesto, Biavati, B. & Mattarelli, P. Microbial community diversity in primates' faeces.

Microbial community diversity in primates' faeces

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INTRODUCTION

The gut of primates could represents a storehouse of microbial diversity in which several known and unknown microorganisms (Michelini *et al.*, 2015a, b; Modesto *et al.*, 2014, 2015) with unexpected functions that may be studied to employ microbes for application in the environmental, pharmaceutical, agricultural or industrial fields are harboured. It is a vast gene pool for biotechnological exploitation and should represent a major challenge to understand the microbial phylogenetic relationship and ecological significance (Agrawal *et al.*, 2010). On the other hand deepen the complexity of bacterial diversity is of particular importance because bacteria may well comprise the majority of earth's biodiversity and mediate critical ecosystem processes (Cavigelli & Robertson, 2000; Torsvik *et al.*, 2002). The microbial biodiversity describes complexity and variability among microorganisms at different levels of biological organization, including genes, species, ecosystems, evolutionary and functional processes that link them (www.for. gov.bc.ca/pab.publctns/glossary/b.htm). Approaches to characterize and classify microbial communities by cultivation methods have switched to the molecular and genetic level, thus as reported by Muyzer (1999) cultivation-based techniques allowed merely a glimpse of microbial diversity as only an estimated 1% of the naturally occurring bacteria has been isolated and characterized so far. In this view a polyphasic approach involving a combination of molecular biology techniques and conventional microbiological methods seems necessary for a better understanding (Das *et al.*, 2014). Taxonomists have been developed several molecular approaches that provide rapid profiling of microbial communities offering information about phylogenetic groups present to the study of the microbial diversity in an environmental sample. Polymerase chain reaction (PCR)- based molecular methods provide a fast and sensitive alternative to conventional culture techniques (Agrawal *et al.*, 2010). Mainly, molecular 16S rDNA-based PCR techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphisms (SSCPs), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphisms (T-RFLPs) and ribosomal intergenic spacer analysis (RISA) can provide detailed information about community structure of an ecosystem in terms of richness, evenness and composition and can be used to compare different species present in a (Rawat & Johri, 2014). The 16S rDNA gene regions, which are highly conserved within closely related taxa (Santos & Ochman, 2004), has been used as a phylogenetic marker for classification of bacteria into different taxa (Singh *et al.*, 2011). Avoiding limitations of cultivability, the categorization of the 16S rDNA gene from the community DNA of environmental samples has become wide popular as an alternative to characterise microbial communities directly providing information on phylogenetic diversity (Zhou *et al.*, 1997). The use of this technique required cloning and sequencing strategies, which are cost, time and labour consuming for the monitoring a large number of samples. In the 1993 Muyzer *et al.* introduced the denaturing gradient gel electrophoresis (DGGE) of the PCR amplified DNA fragments in the microbial ecology to study the structural diversity of

microbial communities. DGGE represents a consolidate approach for diversity study that overcomes the disadvantages in cloning and sequencing of the DNA fragments (Singh *et al.*, 2011). ARDRA is based on DNA sequence variations present in PCR-amplified 16S rRNA genes the PCR amplicon from environmental DNA is digested with tetracutter restriction endonucleases, such as *AluI* and *HaeIII*, and restricted fragments are resolved on agarose or polyacrylamide gels (Agrawal *et al.*, 2010). This method provides little or no information about the type of microorganisms present in the sample, but it is still useful for rapid monitoring of microbial communities over time, or to compare microbial diversity in response to changing environmental conditions (Agrawal *et al.*, 2010). ARDRA is also used as sensitive technique with high resolution providing reliable genotypic characterization at the community level of compost bacteria (Heyndrickx *et al.*, 1996). Repetitive element sequence-based polymerase chain reaction (rep-PCR) fingerprinting represents a genotypic technique using outwardly facing oligonucleotide PCR primers complementary to interspersed repetitive sequences, such as evolutionarily conserved repetitive sequences are BOX, ERIC, REP and $(GTG)_5$, which enable the amplification of differently sized DNA fragments lying between these elements (Masco *et al.*, 2007). Rep-PCR fingerprinting is a valuable tool for classifying and typing of a wide range of Gram-negative and sever- al Gram-positive genera (Versalovic *et al.*, 1994). Indeed, the distribution of these repetitive sequences (BOX and ERIC) as nearly a true reflection of genomic structure and amplification of inter- REP elements often detects similarities in a given group of bacteria (Agrawal *et al.*, 2015). BOX elements are repetitive sequences randomly located within the whole genome and by using BOX primers the amplification of genomic regions between the two BOX elements should be performed. Masco *et al.* (2007), testing different rep-PCR methods, has been reported the BOXA1R primer has the most suitable rep primer for the identification of bifidobacteria. Enterobacterial repetitive intergenic consensus (ERIC) sequences are short interspersed repetitive elements found in the genome of eubacteria (Gillings & Holley, 1997) and distributed throughout extragenic regions of the genomes of many gram negative enteric bacteria and closely related phyla (Versalovic *et al.*, 1994). Cause their unique location, ERIC elements in bacterial genomes allows discrimination at genus, species, and even strain level based on the electrophoretic pattern of amplification products (de Bruijn, 1992). Selective amplification of ERIC elements using oligonucleotide primers generates amplicons of varying sizes, ranging from 50 to 3000 bp, which collectively constitute a DNA fingerprint (Di Giovanni *et al.*, 1999a). Comparative studies of electrophoretic fingerprints are used for identification, discrimination and classification of bacterial strains or communities (Ben Amor *et al.*, 2007; de Bruijn, 1992; Di Giovanni *et al.*, 1999b). There are only few literature information on application of such molecular based techniques (BOX- and ERIC- PCR) in studying the microbial communities in environmental sample (Cifuentes *et al.*, 2000; Dunbar *et al.*, 2000; Ennahar *et al.*, 2003; Hobel *et al.*, 2005; Singh *et al.*, 2011). Therefore, the present study tried to investigate the distribution of microbial communities in primates' faeces, such as common marmoset, golden faced saki, ring-tail lemur, black lemur, red-bellied lemur, Alaotran bamboo lemur, grivet, Barbary macaque, cotton top-tamarin and emperor tamarin) by using ARDRA, ERIC- and BOX-PCR fingerprints methods. To improve the microbial diversity analysis in our samples, Real Time PCR was also employed to quantify the abundance of bifidobacteria, lactobacilla and enterobacteria.

MATERIALS AND METHODS

Subjects and fecal samples

In this study, we performed an analysis of the microbial community diversity in 19 healthy subjects primates belonging to ten species at different point of evolution (Table 1), such as five infants belonging to common marmoset (*Callithrix jacchus*) kept in Aptuit s.r.l. (Verona, Italy), and two babies belonging to golden faced saki (*Pithecia pithecia*) and Barbary macaque (*Macaca sylvanus*) respectively, kept in the Parco Natura Viva of Pastrengo (Verona, Italy), and two adult subjects of ring-tail lemur (*Lemur catta*), an adult of black lemur

(*Eulemur macaco*), a red-bellied lemur (*Eulemur rubriventrer*), a Alaotran bamboo lemur (*Hapalemur alaotrensis*), grivet (*Chlorocebo aethiops*), three Barbary macaque (*Macaca sylvanus*), two cotton top-tamarin (*Saguinus oedipus*) and an emperor tamarin (*Saguinus imperator*). Fresh faecal samples from animals were collected and kept at 4°C for up to 6 hours until they were stored at -120°C for further analysis.

Table 1. List of sampled animals and additional information.

Monkeys group	Host Common Name	Host Scientific name	Identification	Age
New World Monkeys	**Common Marmoset**	*Callithrix jacchus*	MRM_5	baby
New World Monkeys	**Common Marmoset**	*Callithrix jacchus*	MRM_6	baby
New World Monkeys	**Common Marmoset**	*Callithrix jacchus*	MRM_7	baby
New World Monkeys	**Common Marmoset**	*Callithrix jacchus*	MRM_8	baby
Lemurs	**Ring-tail lemur**	*Lemur catta*	LMC_A	adult
Lemurs	**Ring-tail lemur**	*Lemur catta*	LMC_F	adult
Old World Monkeys	**Grivet**	*Chlorocebo aethiops*	CRT_B	adult
Old World Monkeys	**Barbary macaque**	*Macaca sylvanus*	BRT_D	adult
Old World Monkeys	**Barbary macaque**	*Macaca sylvanus*	BRT_E	baby
Old World Monkeys	**Barbary macaque**	*Macaca sylvanus*	BRT_G	adult
Old World Monkeys	**Barbary macaque**	*Macaca sylvanus*	BRT_H	adult
Lemurs	**Black lemur**	*Eulemur macaco*	LMM_C	adult
Lemurs	**Black lemur**	*Eulemur macaco*	LMM_I	adult
Lemurs	**Lac Alaotra bamboo lemur**	*Hapalemur alaotrensis*	LMB_L	adult
Lemurs	**Red-bellied lemur**	*Lemur rubriventer*	LMR_M	adult
New World Monkeys	**Saki**	*Pithecia pithecia*	SK_N	adult
New World Monkeys	**Cotton top-tamarin**	*Saguinus oedipus*	TRE_O	adult
New World Monkeys	**Cotton top-tamarin**	*Saguinus oedipus*	TRE_P	adult
New World Monkeys	**Emperor tamarin**	*Saguinus imperator*	TRI_Q	adult

Isolation of microbial community DNA from faeces

The microbial DNA from about 200 mg of frozen faecal material was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with minor change in the modified lysis step proposed by (Junick & Blaut, 2012). Briefly, after homogenization of about 200 mg faeces with 0.750 ml of lysis buffer from the kit by vortexing for 2-5 min in a 2 ml tube containing 0.5 g of sterile zirconium silica beads (0.1 mm in diameter; Roth, Karlsruhe, Germany), the final suspension was incubated in a water bath at 95°C for 15 min. After cooling on ice for 2 min, coarse particles, cell debris, and the zirconium/silica beads were spun down by centrifugation at 15.000 rpm for 1 min, and the supernatant was transferred to a new 2 ml tube. The pellet was mixed with additionally 750 ml lysis buffer, vortexed for 2-5 min, and the lysis step was repeated at the same conditions described above. The recovered supernatants were combined and the DNA extraction was completed following the manufacture's instructions. DNA was stored at -20°C until further analysis; purity and concentration of DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200PRO NanoQuant, Tecan, Mannedorf, Switzerland).

16S rRNA amplification

About 1440 and 523 bp of the 16S rRNA gene were amplified from the crude DNA using the universal primers 27F and 1492R (Wilson *et al.*, 1990) and the genus-specific for bifidobacteria primer pair Bif164 and Bif662 (Langendijk *et al.*, 1995). Amplification was carried out in an Applied Biosystem Verity Thermal cycler (Applied Biosystems, Foster City, CA) with the following temperature profile: initial denaturation at 95°C for 5 min, and 35 cycles with denaturation performed at 94 °C for 60 s, annealing at 58 °C for 50 s, and extension at 72 °C for 90 s; the amplification was completed with a single elongation step (10 min at 72 °C). 20 µl of each PCR mixture contained 1.5 mM of $MgCl_2$, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each deoxynucleoside triphosphate (HotStartTaq plus DNA polymerase MasterMix Kit; Qiagen) 0.1 µM of each primer and 50 ng of DNA template.

The products were stored at -20°C until the analysis. Aliquots (3.5 µl) of each amplicon was electrophoretically separated on a 2% (w/v) agarose gel using a 100 bp DNA ladder (Fermentas) as a molecular marker and followed by ethidium bromide staining. Gels were visualized under UV light. PCR products were purified with NucleoSpin Gel and PCR clean-up (Macherey-Nagel GmbH & Co. KG, Germany) following the manufacturer's instructions.

Amplified ribosomal DNA restriction analysis, ARDRA

Purified PCR products have been used for a double and a single restriction analysis by using the pair of enzyme *BamHI* and *HindIII,* and the enzyme *Mbol* (Fermentas). The restriction mixture contained 10 U of each restrictase (20 U of *HindIII*), 2 µl of appropriate buffer, Buffer BamHI and Buffer R (Fermentas) respectively, 10 µl of the purified PCR product, and distilled water up to 20 µl volume. The restriction digestions was carried out for at 37°C for 1 hour and 30 min and 2 hour and 30 min for PCR product amplified with genus-specific and universal primers, respectively. According to the recommendation of the enzyme producer, the inactivation was carried put at 80°C for 20 min. Restriction products from each sample were separated in 4% agarose gel in TBE buffer. Gels were ethidium bromide stained (0.5 µn/ml) and the restriction patterns visualized under 260 nm UV light (Molecular Imager Gel Doc XR (BIO-Rad).

In silico ARDRA on bifidobacteria 16S rRNA partial gene sequences

Considering all bifidobacteria species retrieved in primates and already validate and putative novel species just isolates in our laboratory, Table 2, two *in silico* double and single restriction analysis, using *BamHI* and *HindIII,* and *Mbol* enzymes, were performed in CLC SequenceViewer program. The entire 16S gene sequences were cut by using the genus-specific primer pair Bif 164 and Bif 622. Almost 500 bp sequences were obtained from each strain.

Table 2. List of bifidobacteria species and strains considered for the study.

Species	Collection Number	Genbank Accession Number
B. aeriphilum	DSM 100196 ^{T}	KU194202
B. aesculapii	DSM 26737 ^{T}	KC807989
B. avesanii	DSM 100685 ^{T}	KU051446
B. biavatii	DSM 23969 ^{T}	AB559506
B. bifidum	DSM 20456 ^{T}	AB437356
B. breve	ATCC 15700 ^{T}	AB006658
B. callitrichos	DSM 23973 ^{T}	AB559503
B. eulemuris	DSM 100216 ^{T}	KP979748
B. hapali	DSM 100201 ^{T}	KP718961
B. lemurum	DSM 28807 ^{T}	KJ658281
B. longum subsp. *longum*	ATCC 55813 ^{T}	AB437359
B. moukalabense	JCM 18751 ^{T}	AB821293
B. myosotis	DSM 10019 ^{T}	KP718942
B. ramosum	DSM 100688 ^{T}	KU051447
B. reuteri	DSM 23975 ^{T}	AB613259
B. saguini	DSM 23967 ^{T}	AB559504
B. scardovii	DSM 13734 ^{T}	JN180852
B. stellenboschense	DSM 23968 ^{T}	AB559505
B. tissieri	DSM 100201 ^{T}	KP718951
MRM 8.19		KP7189459
MRM 9.3		KP7189460
TRE 1		KU254121
TRE 2		KU254122
TRE 34		KU254124
TRE D		KU254126
TRE F		KU254128
TRE H		KU254129

TRE N	KU254130
TRI 3	KU298953
TRI 5	KU298954
TRI 6	KU298955
TRI 7	KU298956
TRI 9	KU298957
TRI 13	KU298949
TRI 15	KU298950
TRI 16	KU298958
TRI 19	KU298959
TRI 24	KU298952
TRI 28	KU298965

Rep-PCR

For the rep-PCR, the crude microbial DNA from each primate faecal sample was amplified using the primer BOXA1R (Masco *et al.*, 2007) and the primer pair ERIC1/ERIC2 (Ventura *et al.*, 2003) for the BOX- and ERIC-PCR respectively.

The 20 µl of final BOX reaction mixture volume contained 1.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each dntps, deoxynucleoside triphosphate, (HotStartTaq plus DNA polymerase MasterMix kit, Qiagen), additional 0.05 mM of dntps, 50 ng of DNA template and 2 µM of primer. The final reaction mixture of the ERIC-PCR, 20 µl, contained 3.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each dntps, deoxynucleoside triphosphate, 1.25 U Taq DNA polymerase (HotStarTaq plus DNA polymerase MasterMix kit, Qiagen), 5% dimethyl sulfoxide, 0.1 mg/ml of BSA (bovine serum albumin) 50 ng of DNA template and 0.2 mM of each primer.

The BOX- (and ERIC-PCR) amplification was performed in an Applied Biosystem Verity Thermal cycler (Applied Biosystems, Foster City, CA), following this temperature profile: initial denaturation step at 95°C for 5 min, 30 cycles of denaturing at 94°C for 3 sec, annealing at 50°C for 1 min (40°C for 1 min) an extension at 65°C for 8 min (72°C for 8 min), and a final extensions step at 65°C for 16 min (72°C for 10 min). The total PCR amplicon from each sample (20 µl) was fractioned by electrophoresis on a 2.5 % agarose gel (w/v) at a voltage of 7 V/cm. Gels were ethidium bromide stained (0.5 µn/ml) and the fingerprinting profiles visualized under 260 nm UV light (Molecular Imager Gel Doc XR, BIO-Rad).

Statistical analysis

The acquired rep-PCR gel images were normalized using GelCompar II software (v 6.5; Applied Maths, Kortrijk, Belgium) and the similarity of resulting banding patterns was assessed by unweighted pair group method with arithmetic average (UPGMA) clustering. The Jaccard correlation coefficient was used for the reconstruction of the BOX, ERIC and combined BOX and ERIC rep-fingerprinting.

Real Time PCR quantification

The abundance of bifidobacteria, lactobacilli and enterobacteria in studied primate species was evaluated in Real Time – PCR. Amplification was performed with the StepOne Real-Time PCR system (Thermo Fisher Scientific) using the three different primer pairs, *xfp-fw* and x*fp-rv* (Cleusix *et al.*, 2010), *F Lacto 05* and *R Lacto 04* (Furet *et al.*, 2009), and *Eco1457-F* and *Eco1652-R* (Frank *et al.*, 2007), for bifidobacteria, lactobacilli and enterobacteria respectively. The 20 μl of amplification mixture containing 10 µl of SYBR Select® Master Mix (Applied Biosystems), 0.4 µM of each primer and 20 ng of DNA. Analysis of PCR amplification and melting curves, used for monitoring the product specificities, was made using a StepOnePlus™ Real-Time PCR System. Conditions included one cycle of 50°C for 2 min, 95˚C for three minutes followed by 40 cycles of denaturation (95˚C) and annealing (50 s) at 61.5˚C, for each primer pair

respectively. The melt curve analysis was run for 40 repeats, increasing increasing the temperature from 60 °C to 95 °C. Measurements were performed in triplicate, and were repeated when variation between measurements exceeded 0.5 CT. The number of bacterial cells per gram of faeces were recognized from amplification data and were transformed to obtain the number, expressed as log colony forming unit (CFU)/g. Standard curves were made by plotting cycle threshold (CT) values, against dilutions of the quantitative standard (*xfp*, and 16S gene PCR fragments) for which the number of gene copies was known. For bifidobacteria, which harbour a single copy of *xfp* gene per cell, the measured CT value was directly proportional to log *xfp* gene copy number and consequently to og_{10} cell number. For lactobacilli and enterobacteria, data were expressed as $\log_{10}$ colony forming unit (CFU)/g, according to the ribosomal RNA (rRNA) copy number available at the rRNA copy number database for lactobacilli and enterobacteria (Stoddard *et al.*, 2015). The amplification efficiency was calculated from the slope of the standard curve using the formula: E=(10-1⁄slope⁄2)•100.

For quantifications, a PCR fragment of the *xfp* gene and the 16S rRNA genes, for bifidobacteria, lactobacilli and enterobacteria respectively, were used as internal standards. Therefore, DNA was extracted from a pure culture of *Bifidobacterium longum* subsp. *longum* ATCC 15708, *Lactobacillus rhamnosus* ATCC 7469vand *Eschierichia coli* ATCC 25922, as above described. Amplification of this DNA was performed with primers descripted above in a Biometra Gradient PCR apparatus (Biometra, Gottingen, Germany) in a 20 µl of PCR mixtures containing 1.5 mM of MgCl2, 20 mM of Tris-HCl, 50 mM of KCl, 200 µM of each deoxynucleoside triphosphate (HotStartTaq plus DNA polymerase MasterMix Kit; Qiagen), 0.1 µM of each primer and 25 ng of DNA template. The conditions for PCR consisted of an initial cycle of 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 1 min, and a final polymerization step of 10 min at 72°C. The PCR product (235 bp) was then purified using the NucleoSpin (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions and photometrically quantified. Different dilutions of purified PCR product were used as a template for the standard curve. The qPCR assays were replicated three times independently.

RESULTS AND DISCUSSION

In this work 19 healthy subjects of primates belonging to ten distinctive species of primates at different point of evolution were considered for a gut microbial and, in particular, bifidobacteria diversity study. Faceal sample were collected from each sampled animal and genomic DNA was extract for the molecular analysis.

Amplified ribosomal DNA restriction analysis, ARDRA

About 1440 and 523 bp of the 16S rRNA gene of both the entire microbial community and the bifidobacteria community were amplified from the crude DNA using universal and genus-specific primer pairs. After purification, the PCR products have been used for the digestions by using the pair of enzyme *BamHI* and *HindIII,* and the enzyme *MboI*.

Restriction performed on the universal gene sequences with the enzyme pair did not give acceptable results (data not show), indeed only few bands of the same size from the amplification of the DNA extract from each animal were separated on the agarose gel. *MboI* enzyme is able to distinguish a more diversity, even if the resolution of the electrophorese run should be improve to highlight the main bands and to make possible a cluster analyses in GelCompar II program.

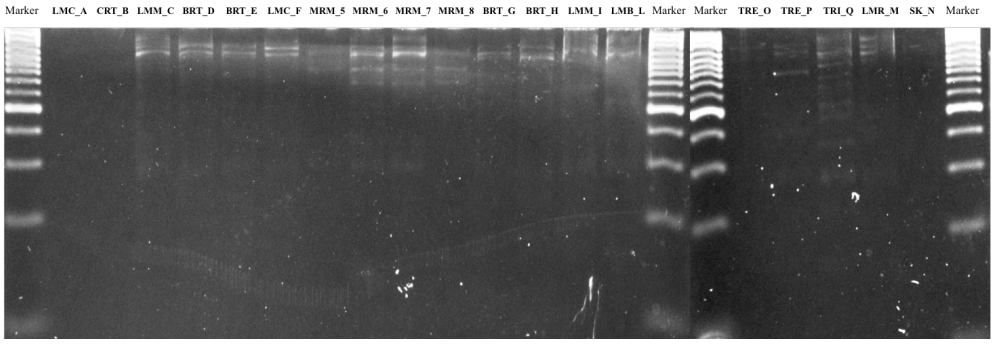
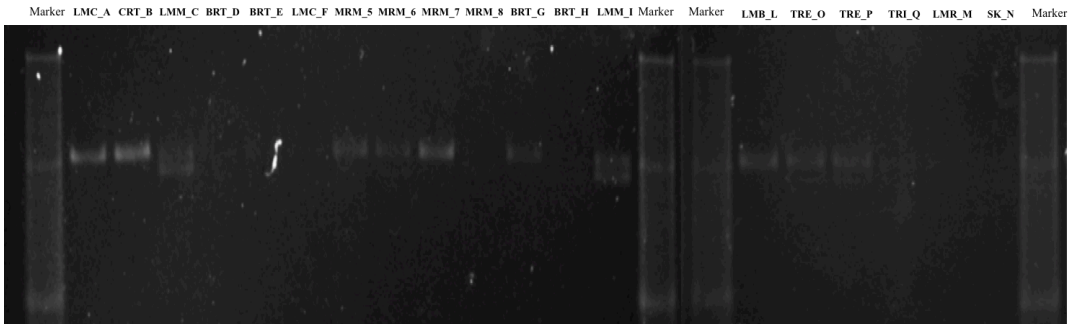


Fig 1. Electrophoretic profiles obtained from the digestion of the about 1400 bp of the 16S rRNA with the enzyme *MboI* for each sampled animal.

Nevertheless, some consideration should be pointed out, even if ARDRA failed for the ring-tail lemur LMC_A and the grivet CRT_B. For example, emperor tamarin, sample TRI_Q, seem to be characterized by a higher diversity, or seem to harbour a peculiar genus or species with many cutting sites, compare to the other hosts, which pattern resulted in numerous bands. A less complexity should be ascribed to the ring-tail lemur LMC_F and the Barbary macaques which profiles are represented by few main bands suggesting the presence of a predominant genus or species.

Considering the bifidobacteria analysis, we performed an *in silico* restriction analyses on the 16S rRNA gene sequences of primate-origin bifidobacteria obtaining 4 and 5 different patterns using the *BamHI* and *HindIII,* and *MboI* enzymes respectively (Fig. 2).

a)



b)

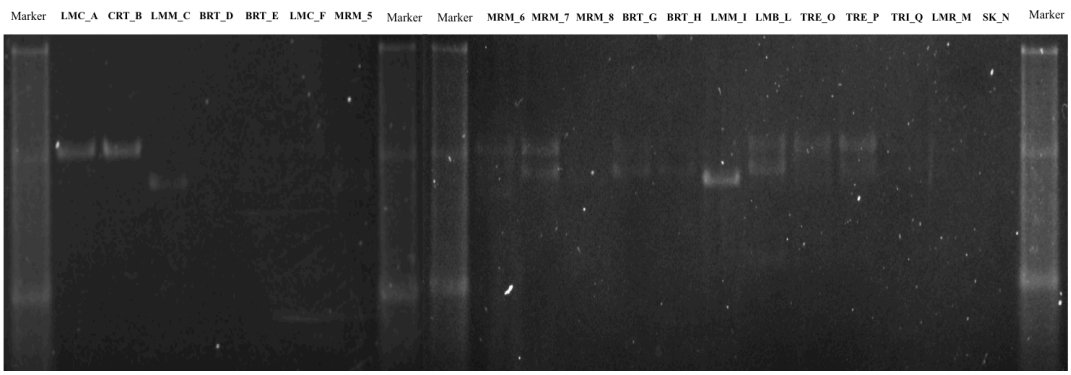


Fig. 2. Electrophoretic profiles obtained from the digestion of the about 500 bp of the 16S rRNA with the enzymes a) *BamHI* and *HindI* and b) *MboI*, for each sampled animal.

Based on the fragments profiles all species and strains were grouped (Table 3 and Table 4) and information was used for the comparison with the *in vitro* work. Unfortunately, digestion with the firs two enzymes seem to fail for samples BRT_D, BRT_E, BRT_H, LMC_F, LMR_M, MRM_8, and SK_N, and no fragments

Table 3. Results from the *in silico* restriction analysis with the enzyme pair *BamHI* and *HindIII*.

Species	***BamHI* and *HindIII***	
	Fragments	**Group**
B. breve B. reuteri	430-67	A
B. lemurum B. hapali B. eulemuris TRI_5 TRI_7	460-42	B
MRM_9.3	290-225	C
TRE_H TRE_D *B. saguini B. longum* subsp. *longum B. aeriphilum*	390-67	D
B. aeriphilum B. aesculapii B. avesanii B. biavatii B. bifidum B. callitrichos B. moukalabense B. myosotis B. ramosum B. scardovii B. stellenboschense B. tissieri MRM_8.19 TRE_1 TRE_ 2 TRE_34 TRE_F TRE_H TRE_N TRI_3 TRI_6 TRI_9 TRI_13 TRI_15 TRI_16 TRI_19 TRI_24 TRI_28	No cutting sites	NCS

Table 4. Results from the *in silico* restriction analysis with the enzyme *MboI*.

Species	***MboI***	
	Fragments	**Group**
B. avesanii	268-160-70	E
B. biavatii B. bifidum B. breve B. callitrichos B. eulemuris B. lemuru B. longum subsp. *longum B. ramosum B. reuteri B. saguini B. scardo B. tissieri* TRE_D TRI_15 TRI_16 TRI_28 TRI_3 TRI_5 TRI_6 TRI_	430-70	F
TRE_H	407-70	G
B. moukalabense	270-225	H
TRE_2	388-70-40	I
B. aeriphilum B. aesculapii B. hapali B. myosotis B. stellenboschens MRM_8.19 MRM_9.3 TRE_1 TRE_34 TRE_F TRE_N TRI_9 TRI_1 TRI_19 TRI_24	No cutting sites	NCS

Table 5. Bifidobacteria groups retrieved from the *in vitro* digestions in each sampled animal.

NCS= no cutting sites; - = no fragments; nc=not considered. In bold species/strains already isolates from the same host are highlighted.

SAMPLED ANIMALS	***BamHI* and *HindIII***	***MboI***	**Bifidobacteria**
BRT_D, BRT_E, MRM_8	-	-	nc
BRT_H	-	F	nc
LMC_F, SK_N	-	NCS	nc
TRI_Q	NCS	-	nc
LMC_A, CRT_B	NCS	NCS	*B. aeriphilum, B. aesculapii, B. myosotis, B. stellenboschense,* MRM_8.19, TRE_1, TRE_34, TRE_F, TRE_N, TRI_9, TRI_13, TRI_19, TRI_24
BRT_G, LMB_L, LMR_M, **TRE_O, TRE_P**	NCS	NCS F	***B. aeriphilum*** *B. aesculapii,* ***B. biavatii****, B. bifidum* ***B. callitrichos****, B. myosotis,* ***B. ramosum****, B. scardovii, B. stellenboschense****, B. tissieri,* MRM_8.19, **TRE_1, TRE_34, TRE_F, TRE_N** TRI_3, TRI_6, TRI_15, TRI_16, TRI_19, TRI_24, TRI_28, , TRI_9, TRI_13, TRI_19, TRI_24
LMM_C	NCS B	F	*B. biavatii, B. bifidum, B. callitrichos,* ***B. eulemuris, B. lemurum,*** *B. ramosum, B. scardovii, B. tissieri, B. hapali,* TRI_3, TRI_5, TRI_6, TRI_7, TRI_15, TRI_16, TRI_28
LMM_I	NCS B	NCS F	*B. aeriphilum, B. aesculapii, B. biavatii, B. bifidum, B. callitrichos,* ***B. eulemuris, B. lemurum,*** *B. hapali, B. myosotis, B. ramosum, B. scardovii, B. stellenboschense, B. tissieri* MRM_8.19, TRE_1, TRE_34, TRE_F, TRE_N, TRI_3, TRI_5, TRI_7, TRI_6, TRI_9, TRI_13, TRI_15, TRI_16, TRI_19, TRI_24, TRI_28
MRM_5, MRM_6, MRM_7	NCS B	NCS F G	*B. aeriphilum,* ***B. aesculapii****, B. biavatii, B. bifidum,* ***B. callitrichos,*** *B. eulemuris, B. lemurum,* ***B. hapali, B. myosotis****, B. ramosum, B. scardovii, B. stellenboschense,* ***B. tissieri*** **MRM_8.19,** TRE_1, TRE_34, TRE_F, TRE_H, TRE_N, TRI_3, TRI_5, TRI_7, TRI_6, TRI_9, TRI_13, TRI_15, TRI_16, TRI_19, TRI_24, TRI_28

were generated. In a similar way, electrophoretical image from the digested using *MboI* did not revealed fragments for samples BRT_D, BRT_E, MRM_8 and TRI_Q. The absence of the uncutting fragments in both the digestion should suggested problems during the digestion stage and/ or with the DNA purity or concentration for samples BRT_D, BRT_E, MRM_8 and TRI_Q.

Results from both the in silico and the in vitro analysis were compared and retrieved information about the digested groups present in each animal was presented in Table 5. Hypothesis about the putative bifidobiota harboured from the primates considered in our study are also reported (due to the problem highlighted above, no considerations were done for BRT_D, BRT_E, BRT_H, LMC_F, LMR_M, MRM_8, and SK_N).

ARDRA is an efficient and easy to perform method to deepen the microbial diversity, but improvement should be performed and better enzymes should be considered if the issue of a work is the bifido-diversity study and the discrimination of the bifidobiota at the species level. Indeed, at our condition, too numerous groups were generated and too many species should be ascribed to the same host.

Rep-PCR

Rep-PCR, such as BOX- and ERIC-PCR, represents a method to obtain genomic DNA fingerprint of bacteria characterising a sample. To deepen the faecal microbial diversity of our primates samples, we performed both BOX- and ERIC-PCR and fingerprinting were statistically analysed in GelCompar II software to reconstruct separated dendrograms for each rep-PCR (Fig. 3 and Fig. 4) performed and to obtain a consensus dendrogram integrating results from both BOX- and ERIC-PCR (Fig. 5).

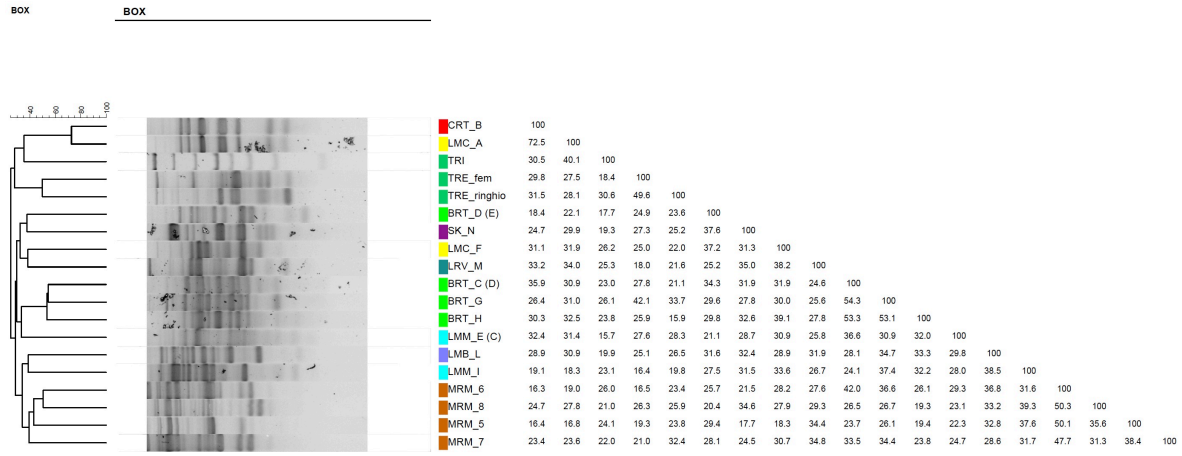


Fig. 3. Dendrogram resulted by the cluster analysis performed in GelCompar II software on the BOX-fingerprinting obtained from each sampled animal. Percentage similarity of banding patterns, as determined by the Jaccard similarity index with optimization and tolerances set to 4 and 1%, respectively, is shown in the x-axis of the tree, while similarity are also reported on the matrix.

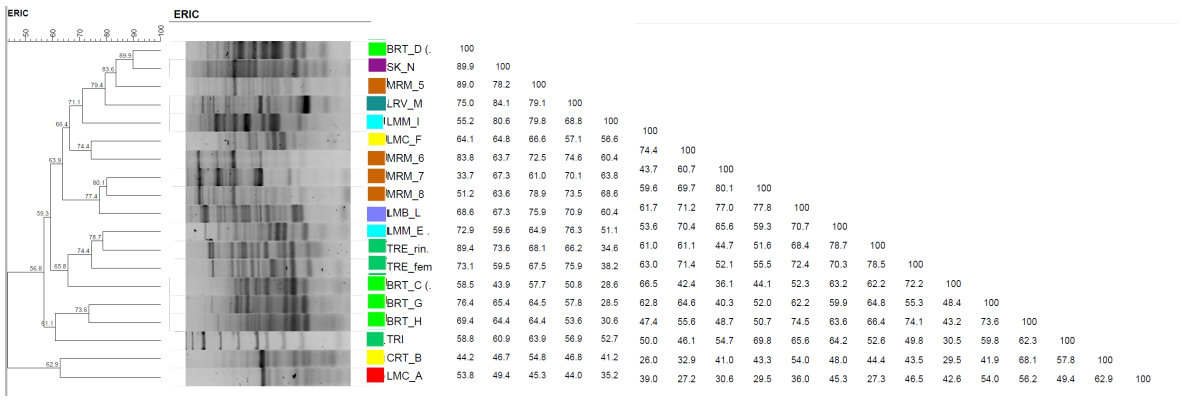


Fig. 4. Dendrogram resulted by the cluster analysis performed in GelCompar II software on the ERIC-fingerprinting obtained from each sampled animal. Percentage similarity of banding patterns, as determined by the Jaccard similarity index with optimization and tolerances set to 4 and 1%, respectively, is shown in the x-axis of the tree.

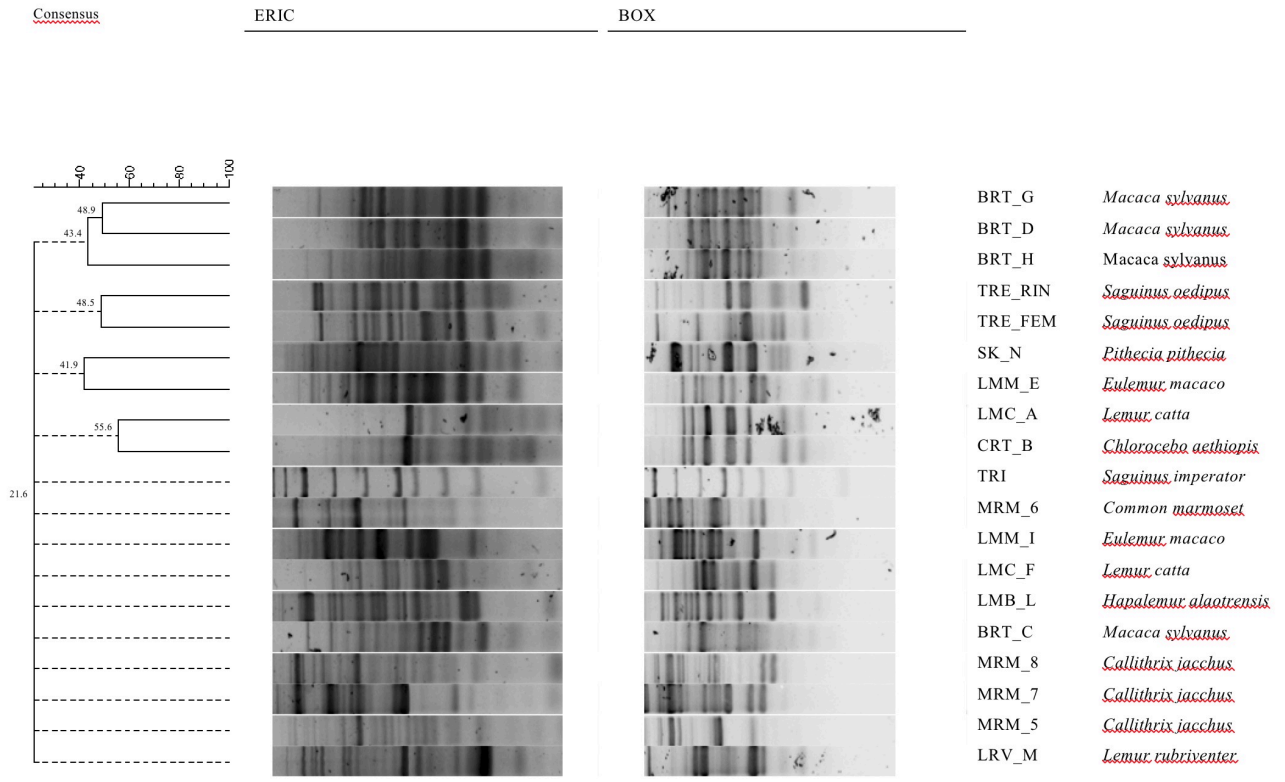


Fig. 5. Dendrogram resulted by the cluster analysis performed in GelCompar II software using the combined BOX and ERIC fingerprint. Percentage similarity of banding patterns, as determined by the Jaccard similarity index with optimization and tolerances set to 4 and 1%, respectively, is shown in the x-axis of the tree, while similarity are also reported on the matrix.

The fingerprint of the ERIC and BOX elements, as well the consensus dendrogram, showed the presence of a high microbial diversity between primates under study Gel compare analysis of the primate-faecal community fingerprints derived from various organisms predominantly microorganisms that are harboured. At our laboratory condition, all samples showed a high number of BOX and ERIC bands, more than 10, suggesting a microbial complexity difficult to be analysed.

Based on the microbial diversity, clustering analysis performed on BOX fingerprinting was able to group together the 3 adult subjects Barbary macaque (53.2%), the 2 subject of cotton-top tamarin (similarity values of 49.65%), and the 4 babies marmoset (39.1%) while the microbial diversity revealed from the analyses of lemurs seem to suggest differences inter and intra species. Surprising the high similarity value (72.5%) was calculated between ring-tail lemur (LMC_A) and grivet (CRT_B).

The cluster analysis performed on ERIC fingerprinting did not reflect the evolutionary history of primates and was not able to found strong similarity between fecal samples collected from some primate species.

The statistical analysis was able to group in some cluster, even if not value did not reach the 90% of similarity, the two cotton-top tamarins (77.4%) and two out of four subjects of both common marmoset (80.1%) and Barbary macaques 73.6%. The baby Saki, SK_N, and the adult Barbary macaque, BRT_D, seem significantly similar showing the highest value of 89.9%, while no strong correlation was found between lemur species which are spread distributed in the dendrogram and appears to be differentially similar to other primates.

The consensus dendrogram confirmed the main clusters already obtained from the rep-PCRs. A particular consideration is to give to the similarity (41.9%) recognized between Saki (SK_N) and the baby Barbary

macaque (BRT_E), which evolutionary belong to the Monkeys group, in particular New World and Old World Monkeys respectively.
Rep-PCR results suggest a very high microbial diversity in primate faecal samples, even if the method is not able to identify a phylogenetic correlation between the host and its microbial community.

Real Time PCR quantification

Results from the Real-Time quantification of the main microbial group, bifidobacteria, lactobacilli and eneterobacteria for each subject under investigation are showed in Table 6.

Table 6. Quantification results of bifidobacteria, lactobacilli and enterobacteria in feces of primates, mean value for subjects belong to the same species and primates group were also calculated.

Sample	bifidobacteria	lactobacilla	enterobacteria
LMC_A	6.11	5.93	8.81
LMC_F	6.55	5.1	7.7
Mean	**6.33**	**5.515**	**8.255**
LMM_C	6.44	5.14	7.03
LMM_I	6.38	5.43	5.27
Mean	**6.41**	**5.285**	**6.15**
LMB_L	5.7	6.39	6.39
LMR_M	6.81	6.47	6.96
CRT_B	7.58	7.55	9.7
BRT_D	6.41	7.87	6.08
BRT_G	6.87	6.91	7.74
BRT_H	7.04	8.02	9.92
Mean	**6.8**	**7.6**	**7.9**
BRT_E	5.67	5.33	5.29
MRM_5	5.86	4.79	3.39
MRM_6	6.13	4.58	3.94
MRM_7	7.98	5.85	5.53
MRM_8	5.44	4.07	3.82
Mean	**6.35**	**4.82**	**4.17**
TRE_O	9.31	4.86	6.32
TRE_P	9.73	5.64	6.34
Mean	**9.52**	**5.25**	**6.33**
TRI_Q	8.69	5.9	5.88
SK_N	6.74	5.83	6.76

Primates group	bifidobacteria	lactobacilla	enterobacteria
Lemurs	6.31	5.92	6.94
Old world monkeys			
Grivet	7.58	7.55	9.70
Barbary macaque	6.20	6.50	6.60
Mean	**6.89**	**7.03**	**8.15**
New world monkeys			
Saguinus spp.	9.31	5.41	6.22
Callithrix spp. (baby)	6.35	4.82	4.17
Pithecia spp. (baby)	6.74	5.83	6.76
Mean Baby	6.55	5.33	5.47

Bifidobacteria quantification. Comparing the values with those from human adult and from infants (6 weeks/3 months), which were esteemed by Junick & Blaut (2012) and of about 10.1 and 9.6/11.1 $\log_{10}$/g of faeces, quantifications of bifidobacteria in our primate faecal samples were generally medium-high. Indeed, values ranged between 5.44 $\log_{10}$/g of faeces, the lowest value obtained from common marmoset MRM_8, and 9.73 $\log_{10}$/g of faeces, recognized in the cotton-top tamarin TRE_P. The cotton-top tamarin is the species that harboured the high bifidobacteria quantity, mean value of 9.52 $\log_{10}$/g, followed by the emperor tamarin, 9.52 $\log_{10}$/g. These significant counts for tamarin are in agreement with the literature; in their study Endo et al. (2010) have been reported a value of bifidobacteria of about 8.52 $\log_{10}$/g in adult subjects of red-hand

tamarin. These results are one/two-fold higher than those recognized from the other primate species. As well documented, the colonization of the gastrointestinal tract starts at birth and is characterized by different microbial successions (Junick & Blaut, 2012). In their study on humans Junick & Blaut (2012) found absence or low concentration of *Bifidobacterium* species at the first week after birth, while at 3 months of age they became one of the dominant population groups in the infant gut. In contrast with human infants, in baby primates counts were lower. The number of 6.34 $\log_{10}$/g obtained for the baby common marmosets is almost 3-fold lesser than in adults subjects, 9.11 and 8.52 $\log_{10}$/g in common marmoset and tamarin respectively (Endo *et al.*, 2010). Our quantification revealed a similar trend in Barbary macaques, were adults subjects harboured a bifidobacteria mean value of 6.80 $\log_{10}$/g, while the baby of 5.67 $\log_{10}$/g. Low cell-count were measured in lemurs, mean value of 6.31 $\log_{10}$/g, while the grivet showed a medium number of 7.58 $\log_{10}$/g.

Lactobacilli quantification. Lactobacilli were enumerated in all primate species and were found more concentrated in two out of three adult subjects of Barbary macaque, BRT_H and BRT_D (8.02 and 7.87 $\log_{10}$/g of faeces respectively), followed by grivet (7.55 $\log_{10}$/g). Lactobacilli were commonly present in medium amount, ranging between 4.86 and 6.47 $\log_{10}$/g of the cotton-top tamarin TRE_O and the red-bellied lemur, respectively. The lowest values were obtained from the baby common marmosets (mean value of 4.82 $\log_{10}$/g) and in particular from subject MRM_8 (4.07 $\log_{10}$/g). A similar quantification was reported by Endo et al. (2010) in adult subject of *Callithrix jacchus*, 4.79 $\log_{10}$/g, suggesting the absence of variation between baby and adult animals in common marmoset, while a growth in the lactobacilli amount should be found in Barbary macaque. Indeed, as in bifidobacteria, also in lactobacilli our quantification results revealed an increase of 1/2-fold in the values from baby (5.33 $\log_{10}$/g) to adult (mean 6.5 $\log_{10}$/g).

Enterobacteria quantification. The quantification of *Enterobacteriaceae* performed during this study revealed intra and inter species, but also individual variability. The highest values were recognized from the grivet, 9.92 $\log_{10}$/g, and from the Barbary macaque BRT_H, 9.70 $\log_{10}$/g. This last number differed from those measured from other adult Barbary macaques (mean value of 6.91 $\log_{10}$/g) and the baby (5.29 $\log_{10}$/g). The ring-tail lemur is the species which harbours the high quantity of enterobacteria, mean value of 8.25 $\log_{10}$/g; this result is in contrast with the quantities measured in the other lemurs, which ranged between 6.15 $\log_{10}$/g of the black lemur and 6.96 $\log_{10}$/g of the red-bellied lemur. The baby common marmoset have been registered the lower amount, mean value of 4.17 $\log_{10}$/g.

Based on our partial results and due to the deficiency of the actual literature, no correlation can be identified between the abundance of positive (bifidobacteria and lactobacilli) and negative gut-microorganisms (enterobacteria). Indeed, even if a high amount of bifidobacteria was measured in Saguinus spp., the amount of enterobacteria is on the average with those of the other specie and similarly, the elevate amount of enterobacteria found in grivet and adult Barbary macaques did not cause a decrement in both bifidobacteria and lactobacilli.

CONCLUSIONS

Aim of the present study was to deepen the microbial community diversity primate host species at different evolutionary point using conventional, rapid and low cost techniques, such as ARDRA and rep-PCRs. For this purpose, genomic DNA was extracted from 19 subjects belonging to 10 primate species (lemurs, and new and old world monkeys) and used for further analysis. Two different restriction analyses (ARDRA) were performed on the universal and the genus-specific, for bifidobacteria, 16S rRNA genes. Rep-PCR techniques were also employed and cluster analyses were performed on the BOX and ERIC elements. Regarding ARDRA, our work underlined the potential of the restriction analyses on the bifidobacteria 16S

rRNA partial gene sequence; indeed it is able to detect *Bifidobacterium* spp. in the total microbial DNA from faecal sample and, with modifications regarding the enzymes, seem able to distinguish the harboured bifidobacteria at the species level. Rep-PCR methods appear the best method to have a look on the community diversity, even if no additional information about family, genus or species should be made. Cluster analysis on both single and consensus fingerprinting seem to not reflect the phylogenetic history of the host and in some case appear to be affected by he individual diversity.

This initial study can pave the way for identify methods for the quick and low cost study of the community diversity in faecal samples, even if improvement should be performed. Optimization should be fixed i) in ARDRA, selecting enzyme with higher discriminatory power at species level for bifidobacteria, ii) in rep-PCR, comparing more then 2-3 subjects for each primates species in order to overcome individual diversity and to emphasize the common set of bacteria which should represented the core set microbiota.

Although, information on the bifidobacteria quantification from more baby and adults subjects should be performed to clear the trend of the presence and the colonization in primates, our partial and primary results on adults should suggest a low presence in evolutionary old primates, such as lemurs and old world monkey, compared to those in more evolute species, such as *Saguinus* spp. and common marmoset. Additionally, in common marmoset and the bifidobacteria concentration in adults and baby seem to be inverted compare to humans. Differently from bifidobacteria, the amount of lactobacilli in faeces of common marmoset seem not diversify according to the age, even if more data should be collected to support the hypothesis. Indeed, in Barbary macaques lactobacilli and enterobacteria seem reflect the same trend of bifidobacteria increasing from baby to adult subjects. Results from our study did not support a correlation between the abundance of positive (bifidobacteria and lactobacilli) and negative gut-microorganisms (enterobacteria).

Further and depth studies are needed to support the hypothesis of variation in the amount of the man microbial groups from baby to adults and to verify if a correlation between bifidobacteria and lactobacilli and enterobacteria could subsist.

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DRAFT 4

Michelini, S., Modesto, Biavati, B. & Mattarelli, P. Bifidobacteria-primate cophylogenetic analysis.

Bifidobacteria- primate cophylogenetic analysis

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ABSTRACT

The evolution of the gut microbiota could be linked to the evolution of their hosts and, on the other hand that speciation by the hosts could cause speciation of their parasites. Previous studies support the hypothesis of co-evolution between bifidobacteria and their hosts, so should be interesting to deepen the cophylogenetic events which occurs between bifidobacteria and primates. In this work, the host-bifidobacteria coevolution was studied by different tree-based methods, TreeMap, Jane and Core-PA, and global-fit methods, PACo and ParaFit in CopyCat. Not all methods agree that there has been extensive cospeciation in this host-bifidobacteria system. The event-based methods did not find significant congruence between tree topologies, probably as a result of occasional host switching by the bifidobacteria and or due to possible failure to speciate events (parasites not speciating in response to their hosts speciating). However not complete congruence were obtained on the results from the two programs, indeed Jane4 find only 7 cospeciation and 14 failure of divergence events, while 11 and 5 events were respectively retrieved by Core-PA. Both of them hypothesized the major occurance of duplication and host-switch events in the system. On the other hand, the global-fit methods statistically support a global cospeciation between host-bifidobacteria, but not all the individual links in the system are significant. All the programs has been found Saguinus spp. and associated bifidobacteria as the main co-evolutionary sub-system with the strongest and statistically significant links.

INTRODUCTION

A complex microbial community harbors the gastrointestinal tracts of animals and its composition finally reflects the coevolution of microorganisms with their animal host as well as the diet of the host (Ley et al., 2008). Example of co-evolutionary systems could be represented by relationships between hosts and their associated parasites, between predators and pray, or between groups of species (Keller-Schmidt et al., 2011).

In humans, the intestinal microbiota, known as gut microbiota, is one of the most densely populated microbial communities and impacts upon different human functions; however, also the microorganisms are not independent from the host and symbiotic interactions between the various bacteria and their human host could be described as a continuum ranging from mutualism and commensalism through to pathogenesis (Ventura et al., 2012). Generally, Ventura et al. (2012) suggested that the co-evolution between bacteria and their host may lead to the development of commensal relationships, where neither partner is disadvantaged, or to symbiotic relationships where unique metabolic activities or other benefits are provided to both partners.

Microorganisms belonging to the obligate anaerobic *Firmicutes*, *Bacteroidetes* and *Actinobacteria* dominate the adult colonic and faecal microbiota; among *Actinobacteria*, the vast majority (up to 100%) are representatives of the genus *Bifidobacterium* (Riedel et al., 2014). Bifidobacteria are Gram positive anerobic bacteria with high GC content and that typically reside in the gastro intestinal tract of different animals, including both warm blood animal and insects (Biavati and Mattarelli, 2012; Ventura et al., 2014). With their study Milani (2015) supported the hypothesis of co-evolution between host and bifidobacteria. Basing on this assumption, the present work would like to focus the co-phylogenetic analysis on primates and the bifidobacteria species which are harbored. The current available literature on primate-bifidobacteria interaction is very scares and information for study were retrieved from (Biavati and Mattarelli, 2012; D'Aimmo et al., 2012; Endo et al., 2012; Samanta Michelini et al., 2015a; Modesto et al., 2015; Tsuchida et al., 2013; Ushida et al., 2010) and isolations performed in our laboratory on cotton top tamarin (*Saguinus oedipus*) and emperor tamarin (*Saguinus imperator*) (data not already published).

Deepening into the microbiota of non-human primates (NHPs) could represent a crucial step to provide insights into the reflection of their features in humans, indeed, as confirmed by Ley et al. (2008) microbiomes NHPs exhibit a much higher similarity with those of primates than with other animals. Unfortunately, information about the gut microbiota of NPHs are scares and only culture-independent studies could be found about few species, such as gorilla (Frey et al., 2006), chimpanzees (Kisidayová et al., 2009; Szekely et al., 2010; Uenishi et al., 2007), macaque (McKenna et al., 2008), pygmy loris (Bo et al., 2010). More comprehensive surveys of primate gastrointestinal microbiomes (Ochman et al., 2010; Yildirim et al., 2010).

To explore the combined evolutionary history of two groups, the co-evolution between them should be investigate and the cophylogeny represents the study of the relationships between phylogenies of ecologically related groups. Coevolution has been hypothesized to occur in animal species whose parental care enables vertical transmission of whole gut communities, and where the properties of the community as a whole confer a fitness advantage to the host (Ley et al., 2006).

Several methods for the cophylogenetic analysis are currently available and can be broadly categorized in two categories: i) event-based methods (or tree-based) and ii) global-fit methods (or distance-based) (Balbuena et al., 2013)**.** Event-based method search for the most probable coevolutionary history of the associated host-parasite taxa using different approaches, such as character optimization, tree reconciliation of the associated taxa, or assignment of relative costs to the evolutionary events (TreeMap3 and Jane4). These methods consider 4 type of events in the cophylogenetic analysis: codivergence (cospeciation), duplication (independent speciation of the parasite), host switching and extinction which also includes two other loss phenomena, missing the boat (failure to track all host lineages following a speciation) and sampling failure (failure of the researcher to observe parasites on their hosts). Because the incapability to accommodate widespread parasites, parasites associated with

more than one host, these kind of methods are often unsuitable for the co-phylogenetic analysis (Peterson et al., 2010).
The global-fit methods quantify the degree of congruence between two by-user given topologies, and identify the associations contributing to the cophylogenetic structure without explicitly evaluate evolutionary scenarios, even if the amount of phylogenetic congruence can be related to the importance of coevolution in the system studied (Ronquist, 1997). The most utilize program in this category is ParaFit (Legendre et al., 2002) which could be carried out in COPYCAT (Meier-Kolthoff et al., 2007). Thus, in this study we tried to investigate the cophylogenetic pattern in primate host and bifidobacteria system to understand how hosts and bifidobacteria coevolve.

MATERIALS AND METHODS

Data about the association between host and parasites were derived from the literature (Biavati and Mattarelli, 2012; D'Aimmo et al., 2012; Endo et al., 2012, 2010; Samanta Michelini et al., 2015a, 2015b; Modesto et al., 2015, 2014; Tsuchida et al., 2013; Ushida et al., 2010) and an ongoing work at our laboratory (Data not already published).

Primate and bifidobacteria phylogenies.

Table 1. List of primate host.

Monkeys group	Host Common Name	Host Scientific name
Prosimians (Lemurs)	**Ring-tail lemur**	*Lemur catta*
Prosimians (Lemurs)	**Black lemur**	*Eulemur macaco macaco*
New World Monkeys	**Common Marmoset**	*Callithrix jacchus*
New World Monkeys	**Cotton top-tamarin**	*Saguinus oedipus*
New World Monkeys	**Cotton top-tamarin**	*Saguinus oedipus*
New World Monkeys	**Emperor tamarin**	*Saguinus imperator*
Great Apes	**Chimpanzee**	*Pongo troglodytes verus*
Great Apes	**Orang-utan**	*Pan pygmeus*
Great Apes	**Gorilla**	*Gorilla gorilla gorilla*
Great Apes	**Human**	*Homo sapiens*

The primate phylogeny, after selection of interested species (Table 1), was based on a sample of 10,000 ultrametric trees from the 10kTrees Project, version 2 (Arnold et al., 2010), which provides a posterior distribution of phylogenies using Bayesian inference from six mitochondrial (CYTB, COX1, COX2, 12S rRNA, 16S rRNA and a gene cluster) and three autosomal genes (MC1R, CCR5, SRY) for 230 primate species. The consensus tree is a maximum credibility tree.
The phylogeny reconstruction of bifidobacteria with primate origin was performed on the available 16S rRNA gene sequences, retrieved from GenBank and on the 16S rRNA amplified and sequenced from strains isolates in our laboratory, which sequences have been submitted to GenBank (the list of species/strains and GenBanck accession number are showed in Table 2).
Sequence data assembly and analysis was performed using the CLC_Sequence Viewer (version 7.5) program available for Mac OS X (www.clcbio.com). Sequence alignments were performed using ClustalW (Jeanmougin and Thompson, 1998), while the phylogenetic tree reconstruction was carried out in MEGA6 (Tamura et al., 2013). Trees were reconstructed using the maximum-likehood (Felsenstein, 1985) with Tamura-Nei as the substitution model (Tamura and Nei, 1993).

Bootstrap values were computed by resampling 1000. From both host and parasite tree, the distance matrix was also retrieved.

Table 2. List of bifidobacteria species and strains considered for the study.

Species	**Collection Number**	**16S rRNA gene sequences** Genbank Accession Number
B. adolescentis	LMG 10502^T	LC071806
B. aeriphilum	DSM 100196^T	KU194202
B. aesculapii	DSM 26737^T	KC807989
B. angulatum	ATCC 27535^T	NR_036853
B. avesanii	DSM 100685^T	KU051446
B. biavatii	DSM 23969^T	AB559506
B. bifidum	DSM 20456^T	AB437356
B. breve	ATCC 15700^T	AB006658
B. callitrichos	DSM 23973^T	AB559503
B. catenulatum	DSM 16992^T	NR_041875
B. crudilactis	LMG 23609^T	KJ463395
B. dentium	ATCC 15423^T	M58735
B. eulemuris	DSM 100216^T	KP979748
B. fecale	JCM 19861^T	NR_133982
B. hapali	DSM 100201^T	KP718961
B. lemurum	DSM 28807^T	KJ658281
B. longum subsp. *infanis*	ATCC 15677^T	M58738
B. longum subsp. *longum*	ATCC 55813^T	AB437359
B. moukalabense	JCM 18751^T	AB821293
B. myosotis	DSM 10019^T	KP718942
B. pseudocatenulatum	DSM 20438^T	JQ805710
B. ramosum	DSM 100688^T	KU051447
B. reuteri	DSM 23975^T	AB613259
B. saguini	DSM 23967^T	AB559504
B. scardovii	DSM 13734^T	JN180852
B. stellenboschense	DSM 23968^T	AB559505
B. tissieri	DSM 100201^T	KP718951
MRM 8.19		KP7189459
MRM 9.3		KP7189460
TRE 1		KU254121
TRE 34		KU254124
TRE D		KU254126
TRE H		KU254129
TRI 5		KU298954
TRI 13		KU298949
TRI 19		KU298959
TRI 28		KU298965

Cophylogenetic analyses

The cophylogenetic analysis was performed on ten host species and 38 strain and species belonging to the genus *Bifidobacterium*. Host and parasite trees and range distribution information were used as input data for the following cophylogenetic analysis by sign different methods, such as: i) topology-based programs (TreeMap3, Jane 4 and Core-PA), ii) event-based programs (PaCo and CopyCat).

i) Event-based analysis.

The cophylogenetic tree-based analysis was performed on Treemap 3.0β, Jane4 and Core-PA. The programs used a tree reconciliation methods which try to map the parasite phylogeny to the host phylogeny. Incongruences are reconciled by attribution to co-evolutionary events (Keller-Schmidt et al., 2011).

In order to test for host–commensals co-divergence in associations between some non-human and human primate species and bifidobacteria, the reconciled trees method as implemented by cophylogeny mapping in TreeMap was used (Charleston and Page, 2002). The method assumes the congruence between host and associate trees based on the common history they share. If trees are incongruent, evolutionary events (cospeciation (C), host-switching (H), duplication (D), and sorting (S)) are hypnotized to make the associate tree congruent with that of the host, such as associate duplication independent of host clado-genesis, associate loss from a host lineage, and associate switching from one host to another. The absence of supported and objective criteria for weighting one kind of event more or less heavily than any other, TreeMap applied the "jungle" method (Charleston, 1998): setting for non-codivergent event (duplication, loss and host switch) cost set to 1 could be choose to generate cophylogenies. In our study, TreeMap 3.0β was used to reconstruct the tanglegram and assess the congruence between parasite and host phylogenies (including outgroups). Unfortunately, TreeMap is not able to analyze and solve much large problem instance (Harrison and Langdale, 2006), such is our host-bifidobacteria association; thus, Tree Map3 was only employed to carry out the tanglegram and provided a graphical visualization of the host-parasite association.

Jane 4 is a software tool for the cophylogeny reconstruction problem. Jane compares the host and parasite tree topologies by optimally mapping the parasite tree onto the host tree using different event costs to find very good, and often optimal, solutions to reconcile the two phylogenetic trees using a polynomial time dynamic programming algorithm in conjunction with a genetic algorithm. For this study we performed the analysis with 2000 generations and a population size of 2000 as parameters of the genetic algorithm. All models were tested using random tip mappings with 100 randomizations. The last event-based program used was Core-PA. It proposes an algorithm for the cophylogenetic reconciliation problem which compute optimal solutions under the relaxation that the solutions can be time inconsistent. Like Jane 4, it carries out an event-based analysis to find the cost minimal reconstructions of cophylogenies history of host parasite systems. In addition, tCore-PA allows for the frequency of events to be evaluated and it is usefull when it is difficult to assign appropriate cost values (Merkle et al., 2010). The phylogenetic trees and information about association between host and bifidobacteria were used as input file. For the reconstruction, the defaults parameters for cost of cospeciation (2), sorting (1), duplication (2) and host switch (3) were preferred.

ii) Global-fit analysis.

PACo, Procrustean Approach to Cophylogeny, is a statistical tool to test for congruence between phylogenetic groups and allowes to multiple host-parasite associations. It is especially appropriate to test the classical co-evolutionary model that assumes that parasites that spend part of their life in or on their hosts track the phylogeny of their hosts (Balbuena et al., 2013). PACo measures the contribution of each individual host-parasite association to the global fit by means of jackknife estimation of their respective squared residuals and confidence intervals associated to each host-parasite link. ParaFit is another global-fit method. We use CopyCat, a tool which provides an easy and fast access to cophylogenetic analyses and which supports the programs AxParafit and AxPcoords, which are highly optimized versions of ParaFit and DistPCoA, respectively (Meier-Kolthoff et al., 2007). For the ParaFit

analysis, 999999 permutation and the Caillienz method was used to correct negative eigenvalues. The presence of global congruence between host and parasite phylogenies is statistically tested as well as the significance of individual host-parasite associations (Legendre et al., 2002).

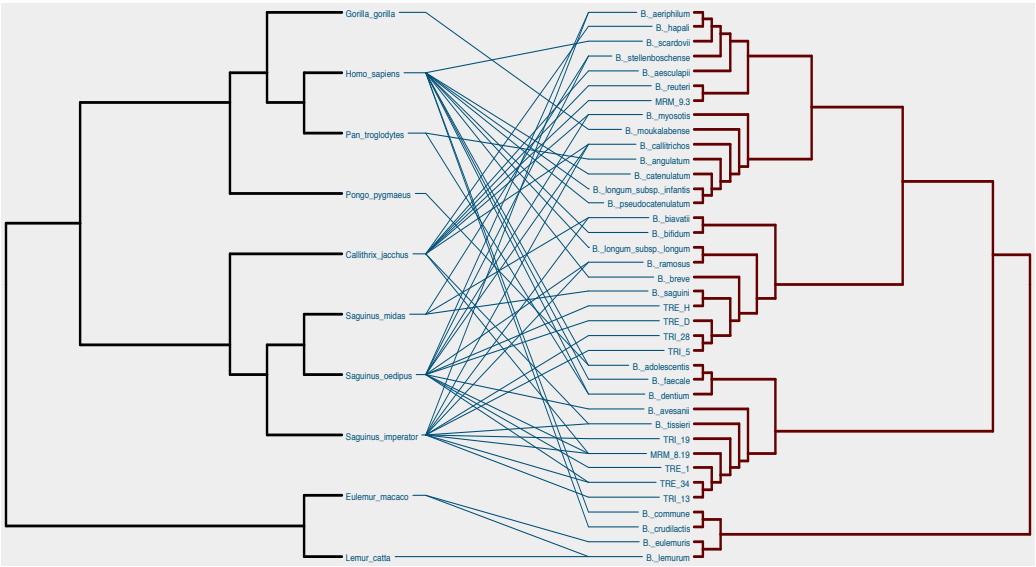
RESULTS AND DISCUSSIONS

Primate and bifidobacteria phylogeny.
The primates phylogeny was retrieved from the 10kTrees Project, version 2 (Arnold et al., 2010), which provides the nexus file format tree for selected primates. The bifidobacteria phylogeny reconstruction was carried out based on the 16S rRNA gene sequences and then exported for the further analysis.

Cophylogenetic analyses.
The cophylogenetic analyses carried out in this study involved ten host species and 38 strain and species belonging to the genus *Bifidobacterium*. The reconstructed phylogenetic tree from both primates (host) and bifidobacteria (parasite) and the association between them were used for the cophylogenetic analyses. Event-based and global-fit methods were employed to test the presence of coevolution between bifidobacteria and primates.

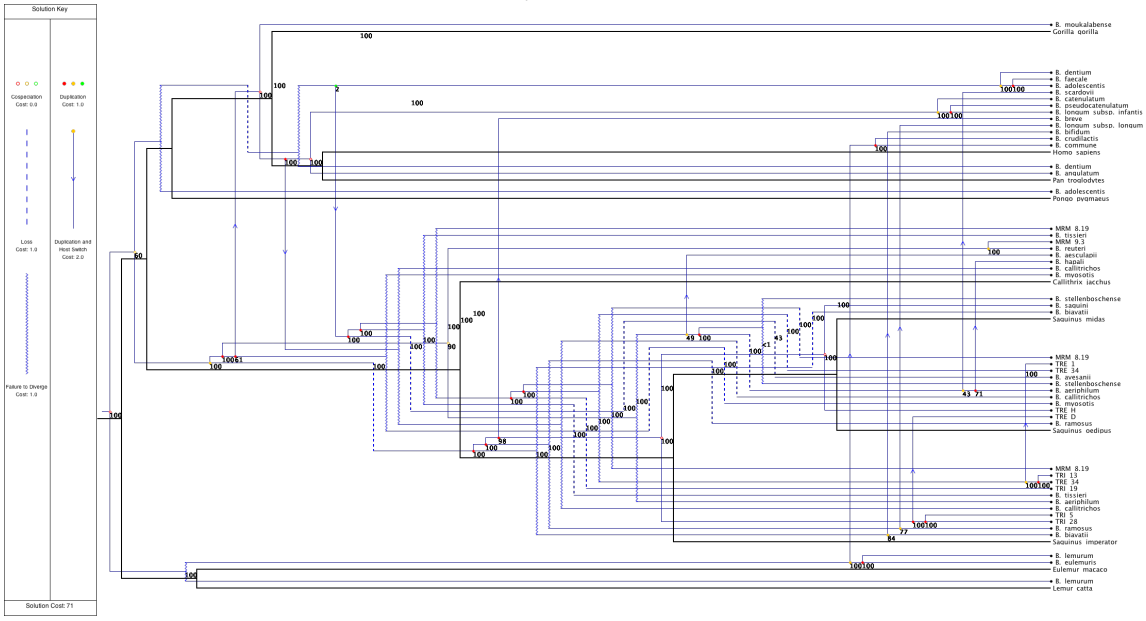
i) Event-based methods
ii)
Treemap 3.0β. Due to the complexity of our data and association, the program was not able to perform the jungle analysis and the program was only employed for the visualization of the tanglegram. The tanglegram built from the phylogenetic trees and individual associations between primate host and bifidobacteria that they harbored is showed in Figure 1 and showed an imperfect match between the two trees. Indeed, the tree topologies may find correspondence with the phylogeny of the host only for some bifidobacteria species, such as *B. lemurum* and *B. eulemuris*, *B. lonum* subsp. *infantis, B. catenulatum* and *B. pseudocatenulatu* and *B. hapali* and *B. reuteri.* Other bifidobacteria seem to be a phylogenetic history incongruent with those of respectively host, such as *B. crudilactis, B. dentium, B. faecale* and *B. adolescentis*.

Figure 1. Tanglegram between bifidobacteria (right) and host (left), lines connect associated host-bifidobacteria species.



Jane 4. We used different cost sets for each of the coevolutionary events to produce different results in Jane 4 (data not showed), but the default parameters were finally preferred based on parameters such as lower quality and total costs (0 for cospeciation, 1 for duplication, 2 for duplication and host switch, 1 for losses and 1 for failure to divergence). The results (Table 3) obtained suggest a total cost of 75 obtained from 3 scenarios, which are the same pattern and suggest 7 event of cospeciation, 18 of duplication, 12 of duplication and host switch, 15 of losses and 14 failure of divergence (in Figure 2 the output of the visualization of the best solution is showed). Globally, Jane 4 did not find a strong cospeciation in the system, indeed losses and duplications are the main events in the reconstructed scenarios. Considering the cospeciation events, the stronger and older could be the coevolutionary of *B. avesani* with its host, *S. oedipus*, but also the *B. tissieri* and TRI_19 with *S. imperator*. More recent could be the cospeciation occurs between the undescribed species TRE_34 and MRM_8.19 and the species *B. callithricos, B. myositis, B. ramosum* and *B. aeriphilum* and their host, *S. oedipus*. The most recent cospeciation occurred may involve *B. biavati* and *S. midas*. Other ancient cospeciation events seem have been characterized bifidobacteria living in the *Saguinus* spp. gut, but finally these failure to diverge. Duplications and host switch events seem to be relatively recent and they mainly affects two host species belonging to the same genus, *S. oedipus* and *S. imperator*, suggesting a most dynamicity in close related species. In different evolutionary time, TRE_34 and TRI_28 may duplicate in TRE_1 and TRE_D, respectively and changed host from *S. imperator* to in *S. oedipus*. While *B. ramosum*, which duplicated in *B. longum* subsp. *longum*, move from *S. oedipus* the most evoluted *H. sapiens*. *B. aeriphylum* duplicated in *B. scardovi* and *B. hapali* moving from *S. oedipus* to *H. sapiens* and *Callithrix jacchus* respectively. *B. biavatii* change host (from *S. midas* to *H. sapiens*) duplicating in *B. bifidum*.

Figure 2. Dendrograms of the best solution obtained from Jane 4. All events could be analyzed using the "Solution key" on the left.



Events of duplication and host switch may be involve also two evolutionary distant primates, such as Lemurs and *H. sapiens*: the common ancestor of *B. lemurum* and *B. eulemuris* could have duplicated to the ancestor of the human bifidobacteria *B. commune* and *B. crudilactis***.** Surprising, in contrast with the evolution, a switch seem that has occurred from human to *S. oedipus* with a duplication of the ancestor of *B. adolescentis*, *B. faecal* and *B. dentium* to *B. avesanii*. Duplication events are relatively newer and mainly affects bifidobacteria harbored by humans, indeed *B. commune*, *B. pseudocatenulatum* and *B. adolescentis* duplicated in *B. crudilactis, B. catenulatum* and *B. faecale* and *B. dentium*, respectively.

Also *S. imperator* harbor a microorganism affected by duplication, such is TRI_28, which duplicated in TRI_5.

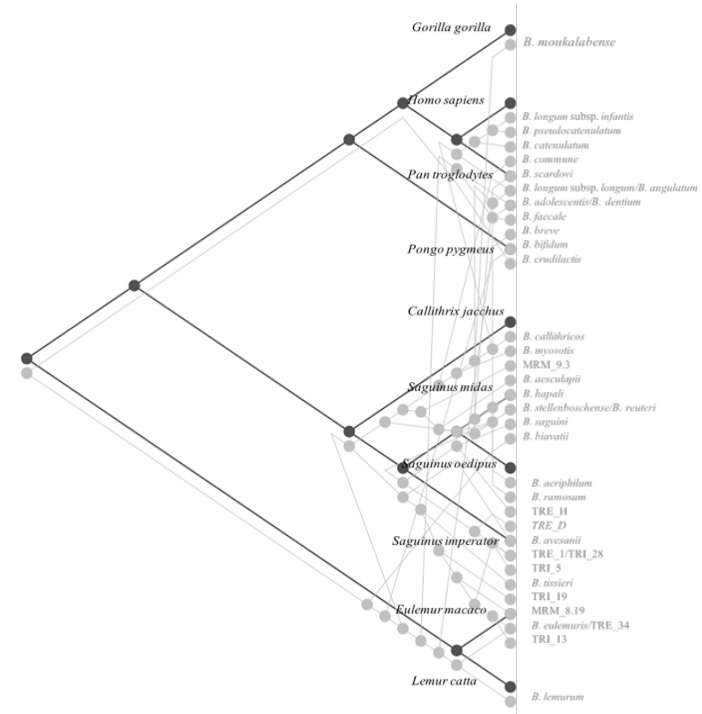
Table 3. Possible cophylogenetic scenarios from Jane4 and Core-PA programs.

PROGRAM	Quality	Cospeciation	Duplication	Duplication and Host switch	Losses	Failure to divergence	Total Cost
Jane4	-	7	18	12	15	14	77
	-	7	18	12	15	14	77
	-	7	18	12	15	14	77
	-	7	18	12	15	14	77
	-	7	18	12	15	14	77
	-	7	18	12	15	14	77
	-	7	18	12	15	14	77
Core-PA	0.619	11	10	16		5	95
	0.619	9	12	16		5	95
	0.619	10	11	16		5	95
	0.667	8	13	16		5	95
	0.71	7	14	16		5	95

Core-PA

Core-PA was used for the reconstruction of the cophylogenetic association between host and bifidobacteria based on their phylogenies. Using default event costs parameters (2 for cospeciation, 1 for sorting, 2 for duplication and 3 for host switch) the program elaborated 5 scenarios. The first three may be the best hypothesis, considering the low quality (0.619) and the relative low total cost (95). Core-PA evaluated a stronger coevolutionary then Jan, identifying more cospeciations (11) and less failure to divergence events (5), which are more time-cost expansive (Table 3).

Figure 3. Reconstruction of the best solution retrieved by Core-PA. Host are in black, bifidobacteria in grey.



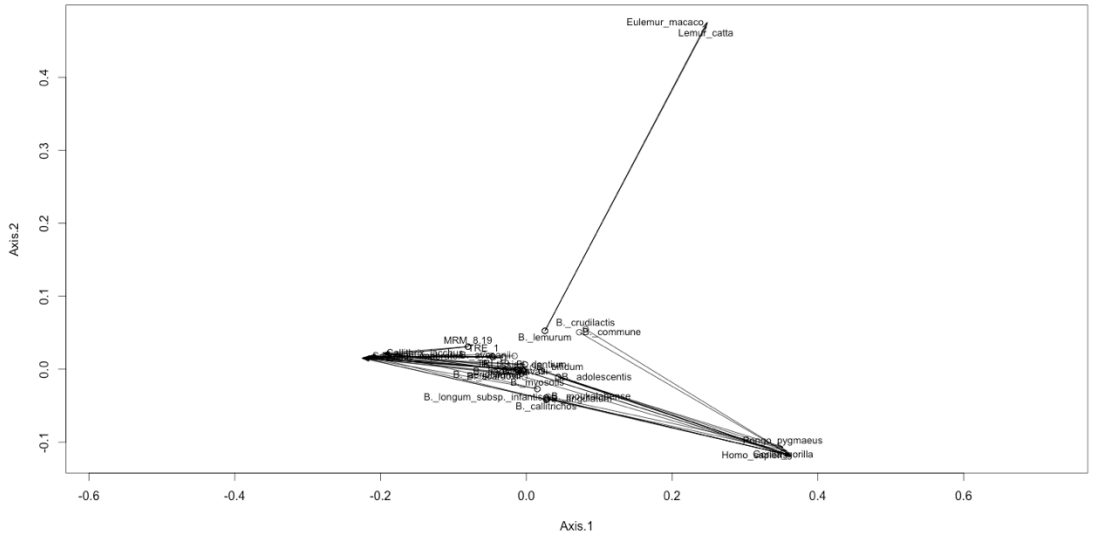
The reconstruction elaborated by Core-PA is showed in Figure 3. The topologies of the trees do not match, but the best association may be involve the primate host lemurs and the tamarins, while most recent may be the cospeciation with humans and chimpanzees and their bifidobacteria. Interesting, there could be identify a possible common bifidobacteria ancestor in cospeciation with lemurs, which could be diversificated in ancestors of other species of bifidobacteria in cospeciation with more evoluted primates. Additional analysis should be carried out to verify the hypothesis and to clarify the evolutionary successions.

iii) Global-fit analysis.

PACo

The global fit method PACo for cophylogenetic analysis based on Procrustes analysis was used because able to statistically tests and quantifies the congruence the between two given topologies (Figure 4) and identifies the host-parasite associations contributing most to the cophylogenetic structure (Figure 5). The analyses indicated that the bifidobacteria phylogram was congruent with the host phylogram (PACo: $m^2_{XY} = 4.611$, p-value $< 10^{-6}$, 10^4 permutations, which supports a co-evolutionary scenario among the host–bifidobacteria association. The contribution of each bifidobacteria-host link to the global fit can be visualized in the Procrustean superimposition plot in which the dots represent the bifidobacteria while the arrow tips are the host; arrow lengths represent the residual sum of squares (m^2_{XY}). Therefore, the shorter the arrows the more likely they are to represent co-evolutionary links. The plot is is able to identify four bifidobacteria cluster which are linked to different host groups. Interesting, among bifidobacteria associated to great apes, *B. crudilactis* and *B. commune* are separated from the other and more close to *B. eulemuris* and *B. lemurum*. Although it is difficult to highlight a separation between the crowd of bifidobacteria, the main groups is charachterized by shorter arrows, which suggest a stronger cospeciation between bifidobacteria and tamarins and common marmoset.

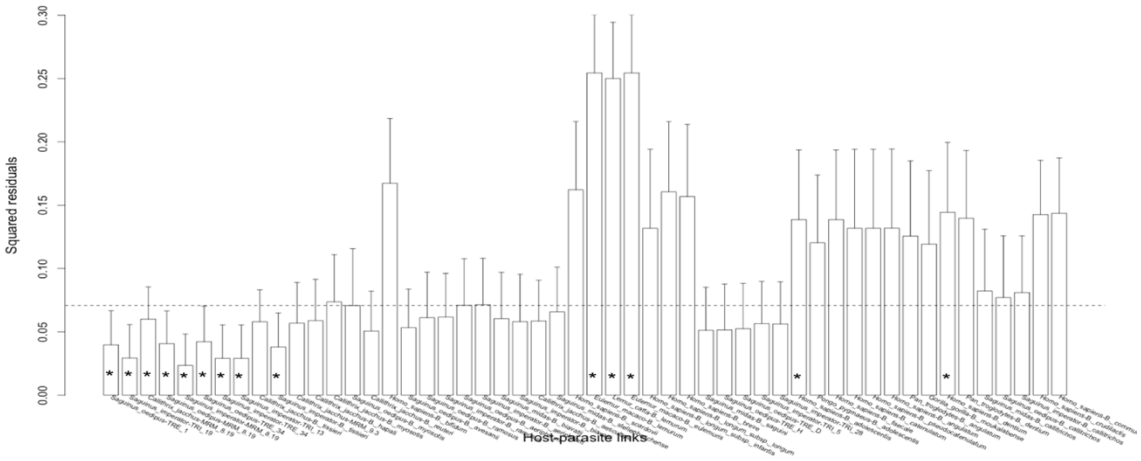
Figure 4. Procrustean superimpostion plot of primates and bifidobacteria used in this study. The ordinations of primates and bifidobacteria are Principal Correspondence Coordinates of patristic distances and the bifidobacteria configuration (dots) has been rotated and scaled to fit the primates ordination (arrow tips).



The bar plot in Figure 5 highlights relative low residual sum of squares, $m^2_{XY,}$ for link relate to the host species *S. imperator, S. midas and S. oedipus* supporting the Procrustean superimposition plot and

suggesting that single links contribute little to m^2_{XY} and may represents co-evolutionary events. Very high values of squared residues were obtained for lemurs and great ape primate species which could be suggested host switching events among rather than host–bifidobacteria co-divergence.

Figure 5. Contributions of individual host-bifidobacteria links to the Procrustean fit. Jacknifed squared residuals (bars) and upper 95% confidence intervals (error bars) resulting from applying PACo to patristic distances. To ease comparisons the median squared residual value is shown (dashed line). Asterisks represent significant links (*p*-value<0.05) evaluated with ParaFit.



CopyCat. CopyCat program was used as tool for a fast and easy cophylogenetic analysis (Meier-Kolthoff et al., 2007); it includes a wrapper for ParaFit. ParaFit conducts a statistical test for the presence of congruence between host and parasite, in this case bifidobacteria. The AxParaFit results have found a high significant overall cophylogeneic structure, ParaFitGlobal of 0.0126 (*p*-value 0.00904, which suggests the existence of cospeciation. Results obtained are summarized in Table 4 and associations are ranked based on their respectively significance. The best association was identify for *B. lemurum* and *B. eulmuris* with their hosts, *L. catta* and *E. macaco* (*p*-value of 0.002). The worst links connecte B. *biavatii* to *S. midas* (0.985) and *S. imperator* (0.983). *S. imperator* and *S. oedipus* are the species with more association harnouring a high bifidobacteria diversity in their gut. The significance of their associations are relatively high, indeed links showed low-medium *p*-values (<0.531) except for associations with *B. callitrhicos* and *B. biavatii* and *B. callitrhicos* and *B. myositis* for *S. imperator* and *S. oedipus* respectively. Considering the human host, even if associations with bifidobacteria showed a medium-low significance (range between 0.046 and 0.840) only the phylogenies of *B. scardovii* and *B. longum* subsp. *longum* are not correlated to the host; while a stronger association involved *B. adolescentis*, and *B. dentium* (p<0.05).

ParaFit significance was used for comparison to support PACo associations squared residuals (Asterisks in Figure 5) when a *p*-value <0.05 characterized the link. Based on this assumption, ParaFit and PACo support the cospeciation between *S. oedipus* and MRM_8.19, TRE_34 and TRE_1, between *S. imperator* and TRE_34, MRM_8.19, TRI_13, TRI_19, TRE_1 and *B. tissieri,* and *C. jacchus* and MRM_8.19. In addition and in contrast with PCAo, ParaFit was able to identify a strong correlation between *B. lemurum* and *B. eulemuris* with *L. catta,* between *B. eulemuris* and *E. macaco*, and a minor, but significant association, between *B. adolescentis* and *B. dentium* with *H. sapiens*.

Table 4. ParaFit results from CopyCat. Different colors are used to visualized the significance of individual host-bifidobacteria associations based on the *p*-value evaluated by ParaFit.

ASSOCIATION		
Parasite	Host	*p*-value
B. lemurum	*Eulemur macaco*	0.002
B. lemurum	*Lemur catta*	0.002
B. eulemuris	*Eulemur macaco*	0.002
TRE_34	*Saguinus oedipus*	0.027
MRM_8.19	*Saguinus oedipus*	0.028
TRE_34	*Saguinus imperator*	0.029
MRM_8.19	*Saguinus imperator*	0.030
TRI_13	*Saguinus imperator*	0.031
TRI_19	*Saguinus imperator*	0.032
TRE_1	*Saguinus oedipus*	0.036
MRM_8.19	*Callithrix jacchus*	0.043
B. tissieri	*Saguinus imperator*	0.043
B. adolescentis	*Homo sapiens*	0.046
B. dentium	*Homo sapiens*	0.048
B. dentium	*Pan troglodytes*	0.052
B. adolescentis	*Pongo pygmaeus*	0.054
B. faecale	*Homo sapiens*	0.056
B. tissieri	*Callithrix jacchus*	0.059
B. longum subsp. *infantis*	*Homo sapiens*	0.117
B. catenulatum	*Homo sapiens*	0.117
B. pseudocatenulatum	*Homo sapiens*	0.117
B. breve	*Homo sapiens*	0.127
B. angulatum	*Pan troglodytes*	0.131
B. commune	*Homo sapiens*	0.136
B. moukalabense	*Gorilla gorilla*	0.141
B. avesanii	*Saguinus oedipus*	0.148

ASSOCIATION		
Parasite	Host	p-value
B. bifidum	*Homo sapiens*	0.243
B. crudilactis	*Homo sapiens*	0.263
TRE_D	*Saguinus oedipus*	0.289
TRI_28	*Saguinus imperator*	0.293
B. saguini	*Saguinus midas*	0.293
TRI_5	*Saguinus imperator*	0.295
TRE_H	*Saguinus oedipus*	0.306
B. stellenboschense	*Saguinus midas*	0.426
B. stellenboschense	*Saguinus oedipus*	0.429
B. aeriphilum	*Saguinus oedipus*	0.452
B. aeriphilum	*Saguinus imperator*	0.460
B. ramosus	*Saguinus imperator*	0.531
B. ramosus	*Saguinus oedipus*	0.532
B. reuteri	*Callithrix jacchus*	0.619
B. hapali	*Callithrix jacchus*	0.677
B. scardovii	*Homo sapiens*	0.718
B. longum subsp. *longum*	*Homo sapiens*	0.840
B. aesculapii	*Callithrix jacchus*	0.856
B. myosotis	*Callithrix jacchus*	0.884
B. callitrichos	*Callithrix jacchus*	0.908
B. myosotis	*Saguinus oedipus*	0.922
B. callitrichos	*Saguinus imperator*	0.936
B. callitrichos	*Saguinus oedipus*	0.938
MRM_9.3	*Callithrix jacchus*	0.962
B. biavatii	*Saguinus imperator*	0.983
B. biavatii	*Saguinus midas*	0.985

CONCLUSIONS

Althought cophylogenetic signal is weak or absent in most host- parasite associations that have been studied to date, significant cospeciation has been inferred in systems where host-switching is prevented by the asocial lifestyle of the host and the low mobility of the parasite (Deng et al., 2013). Previous studies hypothesize a co-evolution between bifidobacteria and their hosts (Ventura et al., 2012). In this study, we tried to carefully assessed the host-bifidobacteria coevolution through the tree-based analyses (TreeMap 3.0β, Jane 4 and Core-PA) and global fit analysis (PACo and CopyCat). Literature data about bifidobacteria living in the gut of primates are very scares and only few works are available (Endo et al., 2012; Michelini et al., 2015a, 2015b; Modesto et al., 2015, 2014). Thus we based our analysis on isolation works we are performing in our laboratory on faecal sample of black lemur, cotton-top and emperor tamarins. Despite not significant congruence between tree topologies was found by the event-based analysis performed in Jane, due to duplication, occasional host switching and possible failure to speciate events by bifidobacteria), Core-PA offers an opposite scenario, with more cospeciation events, even if host-switch and duplication are confirmed as the main events. Global-fit methods statistically support a global cospeciation between host and bifidobacteria, even if not all the individual links in the host-bifidobacteria association are significant. Generally, all the programs used for the cophylogenetic analysis were able to identify a sub-system represented by *Saguinus* spp. and associated bifidobacteria, which is characterized by strong and statistically significant links.

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CHAPTER 4

4. APPLICATIONS: pH AND BILE RESISTANCE, ANIMICROBIAL ACTIVITY, EPSs AND FOLATE PRODUCTION

As results of a long time of co-evolution and adaptation, the microbial diversity contributes to the eubiosis status of our gastrointestinal tract. If disturbances occur the stable status could be ruptured with a reduction of beneficial species and an increase of pathogen or opportunistic bacteria able to colonize the gut. The best solution to maintain a stable status is to use probiotics for fighting pathogens and to re-establish the eubiosis status.

Screening numerous strains seems a main prerequisite for targeted probiotic development, suggesting the importance of strain isolation from unexplored habitats, which represent a source of novel function.

During the present study on the occurrence of bifidobacteria in the gut of several non-human primates, ring-tail and black lemurs, common marmoset and cotton-top and emperor tamarins, strains belonging to both know and unknown bifidobacteria species have been isolated. Several novel species were recognized, even if only six are actually described and published (**PAPER 2, PAPER 3, PAPER 4** and **PAPER 5)** and three are under revision (**DRAFT 1)**.

During the process for new species description, information about optimal growth condition, profile of fermentation and genotypic features were recovered. Some isolates which showed peculiar phenotypic traits of potential interest for probiotic application, such as growth at high temperature, aerobic tolerance, haemolytic activity, optimal growth conditions, and have been further characterized for pH and bile resistance, EPSs and folate production.

4.1. pH, BILE RESISTENCE AND ANITMICROBIAL ACTIVITY

As longer discussed in the Introduction (see 1.3. Bifidobacteria as probiotic strains) *Bifidobacterium* spp. are generally considered probiotics. Indeed, bifidobacteria residing in the human intestine are subject to growing interest due to their possible health-promoting properties, also known as probiotic features (Ventura et al., 2014).

Proper *in vitro* studies should be performed to assure the potential health benefits of probiotics prior to undertaking *in vivo* trials depending on the proposed health benefit (WHO, 2006). To reach the colon in a viable state, microorganisms must be able to resist at specific stress conditions throughout the gastrointestinal tract: the main traits that potentially probiotic strains to possess are the acid and bile tolerance because they have to survive in usually acidic environments of food products and in the gastrointestinal tract. The WHO, in the 2006, defined that to ascertain that a

given probiotic can prevent or treat a specific pathogen infection clinical study must be designed to verify exposure to the said pathogen (preventive study), or that the infecting microorganism is that specific pathogen (treatment study).

4.1.1. RESISTANCE TO ACID CONDITIONS

At low pH conditions, bifidobacteria strains act several response, including maintenance of pH homeostasis by H^+, -ATPase, production of NH_3, regulation of global signalling systems, and general stress response (Jin et al., 2012; Sánchez et al., 2006; Waddington et al., 2010). It is not clear acid resistance differs even among closely related bacteria, among species and strains (Broadbent et al., 2010; Revilla-Guarinos et al., 2014) of bifidobacteria have different acid tolerance levels. Since cell membrane is considered the most important natural defence for cells, cell membrane with different properties may result in different levels of environmental tolerance, including acid tolerance (Yang et al., 2015). However, the relationship between the cell membrane and acid tolerance has not been confirmed in bifidobacteria.

4.1.2. RESISTANCE TO BILE

The bile is mainly constituted by bile acids, which are produced in the liver and stored in the gall bladder and which flow from there to the duodenum during digestion, facilitating the solubilisation and absorption of dietary fats (Ruiz et al., 2013).

Our intestine holds a bile salt concentration gradient ranging between 2% and 0.05% (40 mM – 1 mM), which acts shaping the microbial community of human gut due to the toxin effects on unadapted microorganism (Islam et al., 2011). Indeed, the bile has different effects on cell viability: (i) the lipophilic nature of steroid rings disturbs the lipid packaging and disrupts the proton motive force, causing cell death (Kurdi et al., 2006); (ii) weak acids, bile unconjugated forms, can passively diffuse into the cell and, once inside, they are dissociated producing an acidification of the cytoplasm (Sánchez et al., 2013); (iii) bile salt induces oxidative stress in DNA repair mechanisms, alters the sugar metabolism and the protein misfolding (Begley et al., 2005).

Enteric bacteria, such as lactobacilli and bifidobacteria have developed specific mechanism to resist or to tolerate the bile presence and it represent one of the most important features of probiotic bacteria which are able to survive in the small intestine. This intrinsic bile salt resistance capability is strains dependant, even if, several work have been proved the progressively adaptation of bifidobacteria to the bile presence by subculturing sensitive strains in gradually increasing concentration of bile (Burns et al., 2010; Guglielmotti et al., 2007; Noriega et al., 2004).

4.1.3. ANTIMICROBIAL ACTIVITY

The antimicrobial mechanism acts by probiotics on pathogens may be a combination of events, which could involve the production of a specific enzyme(s) or metabolite(s) that act directly on the microorganism(s), or could also cause the body to produce the beneficial action (WHO, 2006).

The mechanism of inhibition could be related to the production of acetic and lactic acids, even if some species excrete antimicrobial substance or bacteriocins with a broad spectrum of activity towards Gram positive and Gram negative bacteria (Biavati et al., 2000). For the first time in the 1990, Meghrous *et al.*, were able to provide evidence that bifidobacteria produce antimicrobial

substances which have the properties of bacteriocins. In the same year, Gibson & Wang described antibacterial activity by a *Bifidobacterium* strains against *Salmonella, Listeria, Campylobacter, Shigella* and *Vibrio* cholerae, suggesting a certain importance of the inhibition mechanisms with regard to protection against gastroenteritis.

4.2. CASE OF STUDY 1. RESISTANCE TO EXTREME CONDITION BY BIFIDOBACTERIA STRAINS FROM PRIMATES

For centuries lactic acid bacteria (LAB) have been used in the preservation of food and in other areas of the food industry (Shu et al., 1999). Nutritional and health aspects of functional foods incorporating probiotic bacteria have received considerable attention; indeed, in the last decades, the focus on probiotics has been increased since, as described by the ethimology of the word, they have health benefits for animal and human life. Most LAB are recognized as being safe for human consumption due to their ubiquitous distribution on the surface of the human body, and in the gut, and because of their long history of safe use in food products (Shu et al., 1999).

The definition most commonly used of "probiotics" is based on work of ILSI Europe and the WHO (FAO/WHO, 2002). The WHO expert group definition states that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". The mainly used microorganisms, but not exclusively, are members of the genera *Lactobacillus* and *Bifidobacterium* because they are normal inhabitants of the intestinal tracts of humans and other vertebrates. There are numerous application areas for their use both in industry and human health, including preservation of foods (Stiles, 1996). An important challenge is represented by the definition of the fundamental information about physiology of the of candidate probiotics required for a rational selection.

The theoretical basis for selection of probiotic microorganisms includes safety, functional (survival, adherence, colonisation, antimicrobial production, immune stimulation, antigenotoxic activity and prevention of pathogens) and technological (growth in milk, sensory properties, stability, phage resistance, viability in processes) aspects (Saarela et al., 2000). Particularly, main desirable characteristics for a probiotic strain include the ability to survive, alive at its site of action and retain its viability at conditions (acid and bile tolerance) mimicking the harsh environment of a healthy human gastrointestinal tract (GIT) (Hill et al., 2014), safety criteria such as the absence of acquired antibiotic resistance genes, as well as the ability for producing antimicrobial substances (FAO/WHO, 2002; Martín *et al.*, n.d.; Ogier & Serror, 2008; Pérez-Pulido *et al.*, 2006).

The effect of the probiotic strain on other members of the intestinal microbiota and importantly on the host are furthermore to be considered; this includes not only positive health benefits, but also demonstration that probiotic strains do not have any deleterious effects. (Saarela et al., 2000). In addition probiotic must have good technological properties so that it can be manufactured and incorporated into food products without loosing viability and functionality or creating unpleasant flavours or textures (Saarela et al., 2000).

4.2.1. AIM OF THIS WORK

The purposed of this work was to investigate the potential probiotic use of some isolated strains from primates and belonging to the novel species *Bifidobacterium aesculapii, Bifidobacterium*

lemurum, *Bifidobacterium myosotis, Bifidobacterium tissieri, Bifidobacterium hapali*, *Bifidobacterium aeriphilum*, *B. avesanii, B. ramosum* and *Bifidobacterium* spp. MRM_8.19. Strains were screened for their ability to survive throughout the passage to stomach, in particular the haemolytic activity, the acid and bile tolerance, and antimicrobial activity were tested.

4.2.2. MATERIALS AND METHODS

Bacterial strains

Thirteen strains, *Bifidobacterium aesculapii* DSM 29373^T, DSM 29374 and MRM_4.8, *Bifidobacterium myosotis* DSM 100196^T, DSM 100217 and MRM_6.10, *Bifidobacterium lemurum* DSM 28807^T, *Bifidobacterium tissieri* DSM 100201^T, *Bifidobacterium hapali* DSM 100202^T, *Bifidobacterium aeriphilum* DSM 100689^T and TRE_26, *Bifidobacterium avesanii* DSM 100685^T, *Bifidobacterium ramosus* DSM 100688^T and *Bifidobacterium* spp. MRM_8.19(Table 6) have been investigated for their probiotic traits. All the strains tested in this work were isolated during the three years of the research activity described in this thesis, from fecal material of primates, such as baby common marmoset (*Callithrix jacchus* L.), ring-tail lemur (*Lemur catta*), and cotton top-tamarin (*Saguinus oedipus*),

For all further analysis, strains from freeze stock were grown in TPY broth, anaerobically incubate at 37°C for 24 hours and sub-cultured twice in same condition, if not clarified.

Species	Strain	Isolation source
B. aesculapii	DSM 29373^T	Common marmoset
B. aesculapii	DSM 29374	Common marmoset
B. aesculapii	MRM_4.8	Common marmoset
B. myosotis	DSM 100196^T	Common marmoset
B. myosotis	DSM 100217	Common marmoset
B. myosotis	MRM_6.10	Common marmoset
B. hapali	DSM 100202^T	Common marmoset
B. spp.	MRM_8.19	Common marmoset
B. lemurum	DSM 28807^T	Ring tail lemur
B. aeriphilum	DSM 100689^T	Cotton-top tamarin
B. aeriphilum	TRE_26	Cotton-top tamarin
B. avesanii	DSM 100685^T	Cotton-top tamarin
B. ramosum	DSM 100688^T	Cotton-top tamarin

Table 6. List of strains tested and respectively information.

Probiotics trait investigated

To test the possibility to use the strains as probiotics, their ability the haemolytic activity, the resistance at low pH and increasing bile salt concentrations, and the inhibition of pathogens were evaluated.

a) Haemolytic activity

Haemolytic activity was determined for each strain tested by surface plating 0.5 ml of overnight cultures on BD™ Columbia Agar with 5% Sheep Blood and incubating at 37 °C under anaerobic conditions for 48 h. After the time plates were examined for haemolysis.

b) Acid tolerance

Strains capability to survival at low 2, 2.5, 3 and 7 (control) pH was tested. Overnight cultures were pelleted by centrifugation (6000 rpm for 20 min) and washed twice in phosphate buffer saline, PBS, and resuspended in PBS at the same initial volume. 1% of each resuspended culture was inoculated in PBS adjusted at each pH values using HCl 1M. After 0 and 2 hours of aerobic incubation at 37°C, survival population was enumerated by drop plate method. In detail, serial dilutions of the samples were performed in NaCl 0.09% and 10 µl (in triplicate) of each dilution was inoculated into a TPY agar plate. The number of cells was enumerated after 24-48h of incubation at 37°C in anaerobic conditions. The survival rate was calculated following the equation:

$$\text{Survival Rate \% (SR\%)} = \frac{\log_{10} Nt}{\log_{10} N0} \times 100$$

where Nt represents the total viable count of probiotic strains after treatment by PBS or simulated gastrointestinal juices and N0 represents the total viable count of probiotic strains before treatment.

c) Bile tolerance

The resistance of strains under high bile-salt condition was detected at 0.125%, 0.250%, 0.5% and 0.75% of oxgall (Sigma) and without bile (control), in TPY broth. The procedure used is the same of that one used for the pH. Briefly, after pelleted, washed twice and resuspended,1% of each culture was inoculated in TPY at different bile salt concentrations. After 0 and 2 hours of aerobic incubation at 37°C, survival population was enumerated by drop plate method. Plate were incubated at 37°C. The number of cells was enumerated after 24-48h of incubation at 37°C in anaerobic conditions. The SR was calculated as given above.

d) Inhibition of pathogens

The antimicrobial activity of strains was performed using on a representative group of intestinal pathogens: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* M19, *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* ATCC8043 and *Candida albicans* ATCC48274. For the first 4 pathogens, an aliquot from each frozen stock was revitalized in 10 ml of LB broth and incubated 24 hours at 37°C in aerobic condition, while *C. albicans* was put into 10 ml of YPD (Yeast Potato Dextrose) and incubated aerobically at 30 and 37°C. *C. albicans* becomes virulent when incubated at 37°C.

4.2.3. RESULTS AND DISCUSSIONS

a) Haemolytic activity

Haemolytic assay for each strain was performed in BDTM Columbia Agar 5% Sheep Blood. After 48 hours of incubation, the plates were examined. All the strains did not show haemolytic activity and were selected for the further analysis.

b) Acid tolerance

No growth was obtained at pH 2, while no results were achieved from *B. aeriphilum* TRE_26 and *B. ramosum* strains. Viable count and survival rates are displayed in Table 7.

Only some strains were able to survive at pH 2.5, such as *B. aesculapii* DSM 29373^T, *B. myosotis* DSM 100196^T and MRM_6.10, and *B. hapalii* DSM 100202^T. The type strain of *B. aesculapii* showed the highest survival rate, SR=52.2%. All strains survived after 2 hours of exposure at 3 pH. *B. aesculapii* DSM 29374 and *B. myosotis* DSM 100217 are the most resistant, with a SR of 99.2 and 93.5% respectively. *B. myosotis* DSM 100196^T and *B. avesanii* DSM 100685^T are the most sensitive showing the lowest survival rates, 36.8 and 26.0% respectively.

Species	**Strain**	Viable counts after 2 h in PBS at pH 7 (control), 2.5 and 3 (log CFU/mL)						
		Control	2.50	Dev. St.	**SR%**	3.00	Dev. St.	**SR%**
B. aesculapii	DSM 29373^T	6.62	3.48	0.00	**52.2%**	5.53	0.06	**81.0%**
B. aesculapii	DSM 29374	8.70	0.00	0.00	**0.0%**	8.72	0.06	**99.2%**
B. aesculapii	MRM_4.8	4.48	0.00	0.00	**0.0%**	3.96	0.09	**88.2%**
B. myosotis	DSM 100196^T	8.02	2.75	0.08	**33.7%**	2.98	0.38	**36.8%**
B. myosotis	DSM 100217	7.74	0.00	0.00	**0.0%**	7.38	0.24	**93.5%**
B. myosotis	MRM_6.10	6.82	3.00	0.00	**42.8%**	6.22	0.27	**88.5%**
B. hapali	DSM 100202^T	6.80	3.26	0.24	**48.2%**	6.01	0.30	**85.7%**
B. spp.	MRM_8.19	6.28	0.00	0.00	**0.0%**	5.10	0.20	**78.2%**
B. lemurum	DSM 28807^T	8.59	0.00	0.00	**0.0%**	3.46	0.09	**54.7%**
B. aeriphilum	DSM 100689^T	8.47	0.00	0.00	**0.0%**	5.30	0.01	**66.3%**
B. avesanii	DSM 100685^T	8.52	0.00	0.00	**0.0%**	2.00	0.01	**26.0%**

Table 7. Results from the viable counts after 2 h at 7 (control), 2.5 and 3 pH in term of log10 CFU/mL. The standard deviations and the survival rates (SR) are also showed.

c) Bile tolerance

Strain	Viable counts after 2 h in TPY containing bile salt (log CFU/mL)												
	Control	0.125	Dev St.	**SR%**	0.250	Dev. St.	**SR%**	0.500	Dev. St.	**SR%**	0.750	Dev. St.	**SR%**
DSM 29373^T	8.39	8.23	0.08	**100%**	8.22	0.06	**93.1%**	7.35	0.18	**93.1%**	6.67	0.37	**83.0%**
DSM 29374	6.99	5.52	-	**82.5%**	6.05	0.23	**88.7%**	6.04	0.22	**91.2%**	5.81	0.16	**91.1%**
MRM_4.8	7.93	7.84	0.02	**84.8%**	6.59	0.19	**89.6%**	5.41	0.24	**89.6%**	3.75	0.98	**86.8%**
DSM 100196^T	6.57	4.99	-	**83.7%**	5.75	0.02	**94.5%**	5.74	0.06	**96.9%**	5.48	0.11	**95.1%**
DSM 100217	7.06	5.40	-	**61.0%**	5.78	0.24	**85.8%**	5.65	0.07	**84.8%**	5.27	0.05	**83.2%**
DSM 100202^T	6.42	5.64	-	**84.6%**	4.98	0.26	**72.7%**	4.58	0.39	**80.5%**	4.36	0.12	**79.6%**
DSM 28807^T	6.52	7.07	-	**67.7%**	5.03	0.05	**62.8%**	4.47	0.32	**55.5%**	4.68	0.06	**57.2%**
MRM_8.19	6.18	5.47	-	**94.5%**	4.75	0.14	**80.5%**	4.58	0.17	**87.1%**	4.70	0.06	**85.9%**
DSM 100689^T	7.94	7.88	0.04	**94.0%**	7.63	0.13	**94.0%**	6.43	0.10	**81.0%**	5.28	0.15	**69.0%**
TRE_26	7.70	7.66	0.02	**95.7%**	6.28	0.23	**77.0%**	5.06	0.44	**77.0%**	2.80	0.19	**83.6%**
DSM 100685^T	8.34	8.10	0.14	**94.0%**	6.41	0.26	**74.0%**	3.11	0.55	**39.0%**	2.46	0.73	**34.0%**
DSM 100688^T	7.99	7.71	0.01	**91.0%**	6.23	0.22	**81.0%**	3.08	0.20	**47.0%**	1.85	0.00	**34.0%**

Table 8. Viable counts for each strain, expressed as log CFU/mL, after 2 hours of exposition at 0.125, 0.250, 0.500, 0.750% of bile salt. The standard deviations and the survival rates (SR) are also showed.

The resistance to increasing concentration of bile salt in the growth media was performed on all the tested strains, except for *B. aesculapii* MRM_4.8 and MRM_6.10. For all the others strains, viable counts after 2 hours of exposition at 0.125, 0.250, 0.500 and 0.750% of bile salt are reported Table 8.

All the strains showed a good bile tolerance at 0.125 and 0.250% of concentration. *B. aesculapii* and *B. myosotis* seem to be the most resistant species, indeed all the strains showing more then 83.0% of SR at both 0.500 and 0.750% of bile salt concentration. In particular, the type strain of *B. myosotis* showed a very high tolerance, SR of 96.9% and 95.1% at 0.500 and 0.750% respectively. A strong sensitivity at high bile concentration was recognized for *B. avesanii* and *B. ramosum* strains, with registered value of SR ranging between 39.0 and 47.0% and 34.0% at 0.500 and 0.750% respectively.

4.2.4. CONCLUSIONS

All the strains isolated from primates and tested in this work did not showed haemolytic activity. Suppose their use as probiotic, the ability to survive at the acid condition and at the presence of bile salts in the stomach was evaluated.

The major force driving the choose of a candidate seem to be the resistance at low pH, indeed, almost all strains, except *B. avesanii* and *B. ramosum* types, showed a good survival after exposure at high concentration of bile salt in the growth medium.

2 and 2.5 of pH values represent extreme condition for tested strains and only some of them and belonging to the species isolated from common marmoset *B. aesculapii, B. myosotis* and *B. hapali* showed a tolerance, even if SR are low, ranging between 33.7 and 52.2%. Except for *B. myosotis* DSM 100196^T and *B. avesanii* DSM 100685^T, all the others strains showed a good tolerance at 3 pH. Summarizing the results from both pH and bile resistance analysis, strains belonging to *B. aesculapii, B. myosotis* and *B. hapali* could be further analyzed for additional probiotic traits. Also *B.* spp. MRM_8.19 could require more attention showing medium-high results in both the test performed.

4.3. EXOPOLYSACCHARIDES (EPSs) PRODUCTION

There is an increasing interest linked to the ability of some probiotic bifidobacterial strains to produce exopolysaccharides (EPSs). Indeed, several literature works has recently described the EPSs production capability of strains belonging to the genus *Bifidobacterium* (Andaloussi and Talbaoui, 1995; Audy et al., 2010; Bottacini et al., 2014; Kohno et al., 2009; Prasanna et al., 2013; Ruas-Madiedo et al., 2012, 2007; Salazar et al., 2012; Shaun et al., 2011). Probably, the EPSs produced by these microorganisms are involved in adhesion mechanisms (Ruas-Madiedo et al., 2012).

The finding of new EPSs-producing probiotics could be very interesting in order to guarantee a longer survival of the microorganisms in the gastrointestinal tract, but also for their potentiality in the pathogen control and for beneficial effects on human health (Ruas-Madiedo and de los Reyes-Gavilán, 2005).

However, microbial EPSs seem to be involved also in maintain the survival and the viability of microorganism during the technology food process and storage (Grand et al., 2003; Masco et al., 2003; Stanton et al., 1998), and contribute to rheological properties of fermented food products (Audy et al., 2010). In that way, microbial EPSs could represent a special class of bio-thickeners.

Thickeners are long-chain and high molecular mass polymers able to give thickening or gelling properties when dissolved or disperse in water. They are also used for emulsification, stabilization, suspension of particles, control of crystallization, inhibition of syneresis, encapsulation and film forming secondary effects (De Vuyst and Degeest, 1999). Currently, in food industries,

polysaccharides from plants, seaweeds or animals, are used, but the polymers are not always able to response to quality needed or theological properties require.

EPSs produced by microorganisms could be added as additives or starter cultures could be used for the *in situ* EPSs production during the fermentation process in some diary products, such as yogurt and Scandinavian fermented milk "viili" (Duboc and Mollet, 2001). Indeed, in the last years, the interest in natural thickeners increased since they represent an alternative to the ordinarily thickeners employed in industries. EPSs from microorganisms have the potential to be involved in new and improved products, for example low-milk-solid yogurts, low-fat yogurts and creamier yogurts (Mollet, 1996).

The chemical composition, chain length, and structure of the subunits that form the polysaccharide chains, together with the molar mass and radius of gyration of the EPSs molecule, determine the physical characteristics and thereby their viscosity-intensifying properties (Laws and Marshall, 2001; Ruas-Madiedo et al., 2002; Tuinier et al., 2001).

4.3.1. MICROBIAL EPSS

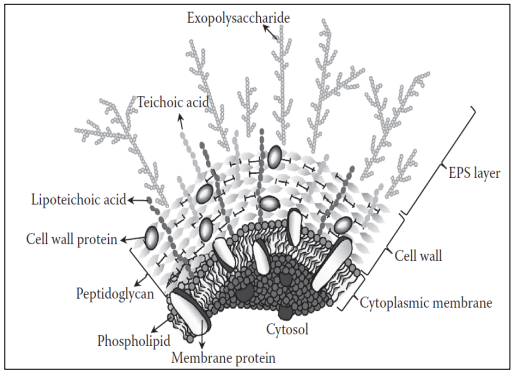

Figure 6. Representation of the Gram-positive cell envelope. (Ruas-Madiedo et al., 2012).

Microbial EPSs are exocellular carbohydrate polymers present as an extracellular layer covering the surface of a vast variety of microorganisms, including Gram-positive as well as Gram-negative bacteria (Hidalgo-Cantabrana *et al.*, 2014).

The main architecture of the enveloped structure of most bacterial cell consisting of a cytoplasmic membrane, a cell wall, and, if present, other external structures, such as the outer membrane in Gram-negative bacteria or layers of polysaccharides or proteins, which could be present either in Gram-negative or Gram-positive bacteria (Ruas-Madiedo et al., 2012). In this latter microbial group, a phospholipid bi-layer membrane forms the cell wall envelope in which some proteins are embedded, surrounded by a thick layer of peptidoglycan, called murein, which is a structural polysaccharide consisting of alternating β-(1→4)-linked N-acetyl- d-glucosamine and N-acetyl-muramic acid residues cross-linked by peptide side chains (Figure 6). Other molecules are associated with peptidoglycan, such as lipoteichoic acids, teichoic acids, and polysaccharides. The polysaccharides include capsular polysaccharides (CPS), which remain attached to the peptidoglycan forming a capsule like in Gram-negative bacteria, and the slime EPSs, which are secreted into the environment (Holts et al., 2009).

4.3.2. CLASSIFICATION, CHEMICAL COMPOSITION AND STRUCTURE OF EPSS

LAB and bifidobacteria are able to synthetized several type of exopolysaccharides, which differ for chemical composition, chain length, molecular size, and structure of the EPSs-forming subunits.

The repeating units are mainly composed by monosaccharides, in which the most abundant are D-glucose, D-galactose and D-rhamnose, derivate monosaccharides, and organic and inorganic substituents. All these features play a crucial role in determine the physical characteristics and thereby their viscosity-intensifying properties (Ruas-Madiedo and de los Reyes-Gavilán, 2005).

a) Classification of EPSs

The EPS produced from LAB and bifidobacteria could be classified, according to their chemical composition and mode of synthesis, as (i) homopolysaccharides (HoPs), if only a single type of monosaccharides is present in the repeating chain. That class could be also divided into 4 subgroups, α-D-glucans, β-D-glucans, fructans and other, such as polygalactan, in pyranose ring conformation, with residues varying in glycosidic linkage and branching degree. The other group is represented by (ii) heteropolysaccharides (HeP), in which several repeating units of different monosaccharides (from two- to octa-saccharides) are comprised (De Vuyst and Degeest, 1999). These two EPS class showed a difference in size, indeed HePs could ranged from 4 to 6 x10^6 Da, while HoPs could be larger and are generally produced in higher quantities than HePs (Cerning, 1995); also their synthesis pathway is different. Indeed, in the HePs biosynthesis a number of genes, organized in *eps* clusters, are involved but the pathway is not clearly understood. Production of HoPs has been described in different genera of LAB, but as far as known, no HoPs-producing bifidobacteria have been reported (Ruas-madiedo et al., 2009; Ruas-Madiedo et al., 2009).

b) Chemical Composition and Structure of Bifidobacteria EPSs

While several studies have been described the chemical composition and structure of EPSs produced by LAB strains, very limited number of polysaccharides extracted from bifidobacteria have been characterized (Shaun et al., 2011).

4.3.3. EPSs Biosynthesis in Bifidobacteria

The priming glycosyltransferase, *p-gtf*, is the key enzyme involved in the catalyses of the first step of the EPSs-units biosynthesis (Hidalgo-Cantabrana et al., 2014). Two putative *p-gtf* were identified in bifidobacteria strains, undecaprenyl-phosphate sugar phosphotransferase, *rfbP*, and galactosyl transferase, *cspD*. The two genes showed a low amino acid homology each other, but the intra-species homology of a single *p-gtf* is very high due to the presence of conserved domains involved in the interaction with the lipophilic carriers (Provencher et al., 2003; Ruas-Madiedo et al., 2007). In previously studies, the important role played by the *p-gtf* gene was confirmed, instead the inactivation of this gene alters or interrupts the production of EPS (Low et al., 1998; Stingele et al., 1996; van Kranenburg et al., 1997).

Although, the EPSs biosynthesis mechanism in bifidobacteria is not known, hypothesis could be proposed based on the functional analysis of few genes and on sequences homology studies, like in biosynthetic pathway proposed for LAB (Hidalgo-Cantabrana et al., 2014).

To underline that, as above mentioned, no HoPs production has been showed from bifidobacteria and HePS are synthesized in a different way from HoPS, because a number of genes, organized in *eps* clusters, are involved in their anabolic pathway; moreover the pathway of HePS synthesis in LAB is not clearly understood (Ruas-Madiedo et al., 2012).

a) The Hypothetical EPSs Biosynthesis Pathway

The pathway of EPSs production in bifidobacteria is not known, but Hidalgo-Cantabrana *et al.* (2014) have been proposed an hypotetical mecchanism based on the funtional analisys of few genes and sequences homology studies.

The EPSs biosynthesis process should be divided into 4 main steps, in which reactions are catalysed by specific enzymes (Hidalgo-Cantabrana et al., 2014).

The biosynthesis begins with the formation of the EPSs-unit. This first step is catalysed by the enzyme *p-gtf,* which transfers a sugar-1-phosphate to the lipophilic carrier molecule anchored to the cell membrane. In the second step, the EPSs-units are build; other *gtfs* are involved in the formation of glycosidic bonds and the transfer of new sugar moieties from the donor nucleotide sugars to the initial monosaccharide of the unit. The followed steps regard the export and the polymerisation process. Briefly, the EPSs-units are moved externally, from the cytoplasm to the extracellular face, by flippase-like proteins. The biosynthesis ends in the fourth step, where specific polymerases assemble the EPSs-units forming the polymers, and chain-length determination proteins stop the elongation.

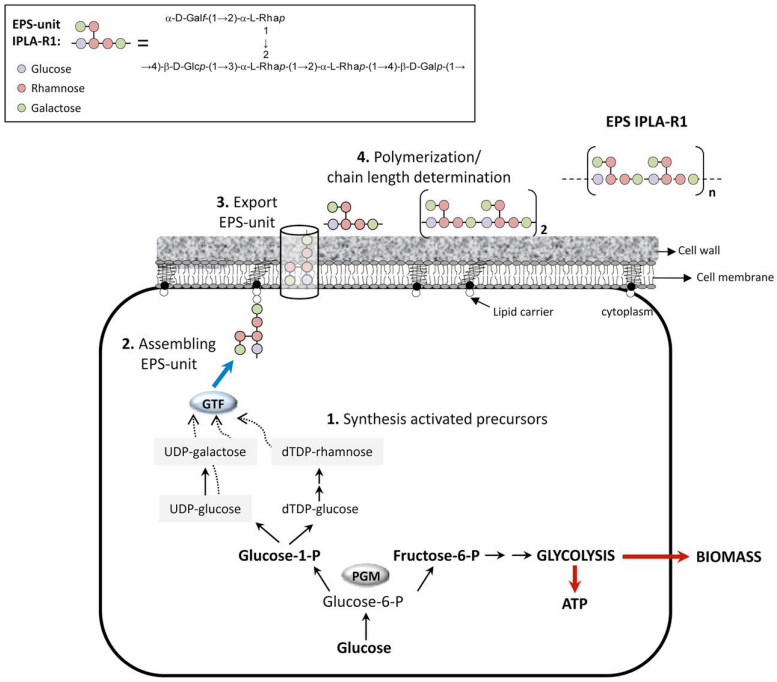

Figure 7. Hypothetical EPSs biosynthesis pathway in the strain *Bifidobacterium animalis* subsp. *lactis* IPLA-R1 proposed by (Hidalgo-Cantabrana et al., 2014). GTF, glycosyltransferase; PMG, phosphoglucomutase.

b) Bifido-EPSs Cluster Genes

Regarding bifidobacteria EPSs production, HePs, and bifido-*eps* clusters, recent studies are mainly based on genomic available sequences, but the functionality, structure and characterization of the retrieved *eps* clusters are not yet clarified.

Genomic sequences of 36 bifidobacteria strains, belonging to 8 species, are available on public database NCBI (see Table 9). As reported in the review by Hidalgo-Cantabrana *et al.* (2014) and summarized in Table 9, genes involved in the EPSs biosynthesis, and comprised in the *eps* clusters, could be located in almost all of them; only *B. breve* (strains PRL2010 and BGN4) and *B. longum* subsp. *longum* (strains DJ010A and JDM301), appeared to be lake, suggesting the ubiquitous of EPSs–producing strains.

The location of *eps* clusters in bifidobacteria is usually executed on the bases of the known LAB *eps* clusters. It is performed *in silico* searching for genes encoding for the enzyme priming-glycosiltransferase (*p-gtf*), which catalysed the first step of the synthesis. Bifidobacteria could show two putative *p-gtf* genes, the undecaprenyl-phosphate sugar phosphotransferase (*rfb_P*) that seems to be harboured in almost all EPSs-producing stains, and the galacotosyl-transferase (*CspD*). These two

enzymes showed a high intra-species amino acid homology, but a low inter-species homology related to the domains in specific sugar of each enzymes.

Once the *p-gtf* localisation, closely genes coding for proteins which are putatively involved in the biosynthesis of HePs, such as *gtf*, export, polymerization and chain length determination, were searching in the surrounding regions (Ruas-Madiedo et al., 2012).

Species	Strain	*eps* clusters	Acc. Numbers	*cspD*	*rfb_P*
B. adolescentis	ATCC15703	+	AP009256	BAD_1389	-
B. animalis* subsp. *lactis	DSM10140	+	CP001606	Balat_1392	Balat_1371
	B1-04	+	CP001515	Balac_1392	Balac_1371
	AD011	+	CP001213	BLA_0595	BLA_0576
	BB-12	+	CP001853	BIF_00944	BIF_00983
	V9	+	CP001892	BalV_1349	BalV_1328
	BLC1	+	CP003039	BLC1_1349	BLC1_1328
	CNCM I-2494	+	CP002915	BALAC2494_01344	BALAC2494_01362
	Bi-07	+	CP003498	W91_1429	W91_1409
	B420	+	CP003497	W7Y_1394	W7Y_1374
	RH	+	CP007755	-	rfb_P
B. animalis* subsp. *animalis	ATCC 27673	nd	CP003941	BANAN_06765	-
	ATCC25527	+	CP002567	-	-
B. asteroides	PRL2011	+	CP003325	-	BAST_1667
B. bifidum	PRL2010	-	CP001840	-	-
	S17	+	CP002220	-	BBIF_0393
	BGN4	-	CP001361	-	-
B.breve	ACS-071-V- Sch8b	+	CP002743	-	HMPREF9228_0447
	UCC2003	+	CP000303	-	Bbr_0430
	NCFB 2258	+	CP006714	nd	nd
	689b	+	CP006715	nd	nd
	S27	+	CP006716	nd	nd
	12L	+	CP006711	nd	nd
	JCM 7019	+	CP006713	nd	nd
	JCM 7017	+	CP006712	nd	nd
B. dentium	Bd1	+	CP001750	-	BDP_1857
B. longum* subsp. *infantis	ATCC15697	+	CP001095	-	Blon_2114
	157F	+	AP010890	-	BLIF_0362
B. longum* subsp. *longum	NCC2705	+	AE014295	BL0237	BL0249
	DJ010A	-	CP000605	-	-
	F8	+	FP929034	-	BIL_15040
	JDM301	-	CP002010	-	-
	JCM 1217	+	AP010888	BLLJ_0364	-
	KACC 91563	+	CP002794	-	BLNIAS_02272
	BBMN68	+	CP002286	BBMN68_1012	-
B. thermophilum	RBL67	+	CP004346	-	D805_0348

Table 9. List of available bifidobacteria whole genomes, presences of *eps* clusters, accession numbers and *p-gtf* genes retrieved by Hidalgo-Cantabrana *et al.* (2014). Nd=not defined

In Figure 8, the physical maps of *eps* clusters detected by Hidalgo-Cantabrana *et al.* (2014) in 7 bifidobacteria strains are showed.

The authors highlighted the lack of a "consensus" functional-structure organisation and an inter-species variability, due to the genome plasticity of the genus *Bifidobacterium*. Indeed, a wide variability in size, localization and number and types of genes characterize the bifido-*eps* clusters. Nevertheless interspecies *eps* cluster length, structure and homology, with few single nucleotide polymorphisms (SNPs), seems to characterize bifidobacteria.

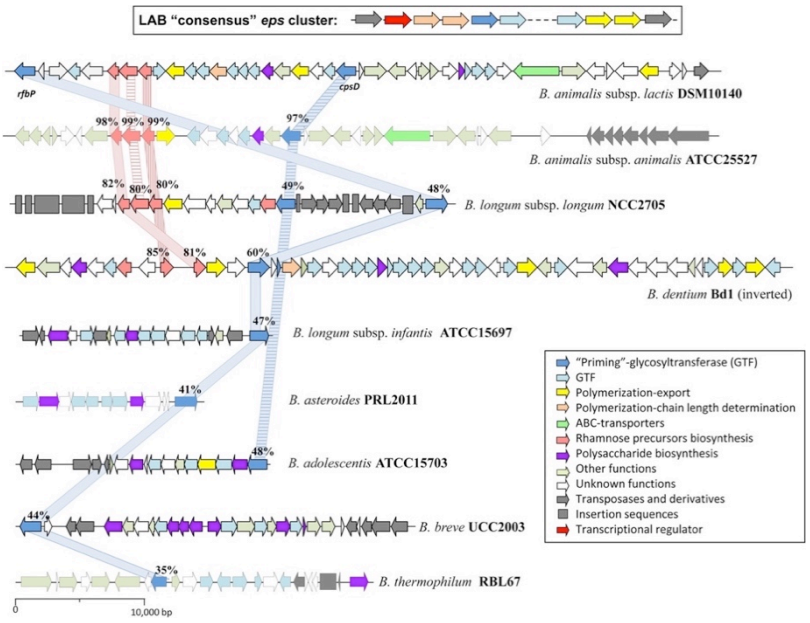

Figure 8. Physical maps of *eps* clusters of 7 species of bifidobacteria and the "consensus" *eps* cluster for LAB (Hidalgo-Cantabrana *et al.* 2014).

4.3.4. Screening and Detection of EPSs-Producing Strains

The improvement of physicochemical properties in fermented food using EPSs-producing bacteria and the EPSs potential as health-promoting effectors have arisen the interest in methods able to screen and detect EPSs producing strains.

As focused in the following paragraphs, a desirable approach for screening EPS production involve both phenotypic and genotypic methods.

a) Phenotypic Methods

Ruas-Madiedo & de los Reyes-Gavilán (2005) reported a compilation of several phenotypic methods for screening, putative EPSs-producing strains.

The authors describe the EPSs-producing bacteria phenotype with two adjectives, (i) ropy and (ii) mucoid, distinguished through macroscopic observation of cultures. In both case, strains could be detected for difficulty in harvested cell by centrifugation (at 2000g for 5 minutes) in liquid cultures, due to the presences of polymers near the surface. Colonies on the surface of agar plates are glistening and smooth aspect, but ropy strains could be distinguished for capability in forming a

filament when a loop is inserted into the colony. The ropy phenotype could be showed also in liquid cultures.

The phenotype of producing strains should be also revealed using optical microscopy, indeed EPSs form a halo around the cells, or using electron microscopy techniques that identified the EPSs as a matrix embedded the cells.

A study by Rawson & Marshall (1997) reveals that ropy phenotypes could confer better technological properties to fermented products.

b) Molecular Methods

Molecular methods for detection of putative EPSs-producing strains involved different polymerase chain reaction protocols in which genes coding for enzymes involved in the biosynthesis were used as target. Many studies have been performed on LAB and specie-specific protocols for detection of definite enzymes are now available, but a method to target *eps* clusters in HePs-producing bifidobacteria has not been established.

Regarding bifidobacteria, HePs-producing bacteria, studies on the *eps* clusters detection *via PCR* are recently introduced and, even if clusters related to the EPSs biosynthesis have been described in available genomes, primers have not been designed for their molecular retrieval and information about their functionality have not been recovered (Ruas-Madiedo et al., 2012). Moreover, *eps* clusters showed a high variability among strains of the same species and it makes difficult designing universal primers.

c) Bioinformatic Tools

Nowadays, the larger amount of public data about bacterial genomic sequences has made easier the studies on EPSs genes involved in the metabolism. Moreover, several bioinformatic tools could be employed in the search for genes involved in specific metabolic pathways, such as EPSs biosynthesis.

Available data about molecular biomedical and genomic information could be retrieved from the National Centre of Biotechnology Information, NCBI: http://www.ncbi.nlm.nih.gov). On this web site researchers could access at different databases that provide information about nucleotide and amino acid sequences and whole or incomplete genomes. The NCBI offers several tools, such as BLAST, Basic Local Alignment Search Tool, to perform search of similar nucleotide, genome or protein sequences, OPF, Open Reading Frame Finder, that finds all open reading frames in a sequence, Primer-BLAST, which uses Primer3 to design PCR primers to a sequence template, e-PCR, Electronic PCR, which identifies sequence tagged sites (STSs) within DNA sequences.

Another helpful web site for retrieved nucleotide information is the European Bioinformatic Institute (EBI: http://www.ebi.ac.uk), part of the European Molecular Biology Laboratory (EMBL). EMBL-EBI provides freely available data from life science experiments, performs basic research in computational biology and offers an extensive user training programme, supporting researchers in academia and industry.

Bioinformatic tools helping researches in design and validate primers are also available.

On the Wubin Qu site, http://biocompute.bmi.ac.cn/CZlab/, different validate instruments could be used for design single or multi primer pairs for multiplex PCR on given sequences, such as VizPrimer or MPRIMER, and software for checking PCR primer specificity *in silico* (allowing degenerate primer pairs), such as MFEPrimer 2.0. *In silico* simulation of molecular biology experiments could be also performed on http://insilico.ehu.es/. This web-server offers tools to perform analysis on prokaryotic genomes or user sequences (PCR amplification, Restriction digestion and PFGE, PCR-RFLP, T-RFLP, Double Digestion fingerprinting, DNA fingerprinting, etc.).

The SIB Swiss Institute of Bioinformatics (http://www.isb-sib.ch/) is an academic, non-profit foundation recognised of public utility; it provides high quality bioinformatics services to the national and international research community. ExPASy is the SIB Bioinformatics Resource Portal which provides several databases (UniProtKB/Swiss-Prot and PROSITE) and tools for helping researchers in many areas, such as proteomics, genomics, structural bioinformatics, system biology, phylogeny/evolution, population genetics, transcriptomics, biophysics imaging, IT infrastructure and drug design.

4.4. CASE OF STUDY 2. DEVELOPMENT OF A PCR-BASED METHOD FOR SCREENING EPSs-PRODUCING BIFIDOBACTERIA

Refers to **DRAFT 5**.

Searching for exopolysaccharides EPSs-producing bacteria is a new challenge in putative probiotic strains selection. Recently, studies on the possibility to screen EPSs-producing bifidobacteria *via* PCR, have gained increased interest and searching for the presence of the priming glycosyltransferase genes seems to be the right choice (Hidalgo-Cantabrana et al., 2015, 2014; Provencher et al., 2003). As set out above, several bifidobacteria strains showed the ability to produce EPSs but the mechanism of their biosynthesis is still hypothesized. Nevertheless, according to Hidalgo-Cantabrana et al. (2014), EPSs-subunits synthesis is initially catalysed by the priming glycosyltransferase, *p-gtf*, which is considered the key enzyme in the EPSs production.

4.4.1. AIM OF THIS SECTION

Another important aspect of the research has concerned the development of an easy and rapid PCR method for the screening of putative EPSs-producing bifidobacteria. This result has been obtained with an *in silico* analysis using bioinformatic tools for designing a primer pair which was subsequently test for target specificity performing an *in silico* PCR on two different web services.

4.4.2. MATERIALS AND METHODS

The method was set-up in two steps: an *in silico* analysis for designing a primer pair for the partial *rfb_P* bifidobacteria gene amplification and an *in vivo* test on bifidobacteria genomic DNA to prove the specificity of three designed primers. All the **Materials and Methods** used for the study are described in **DRAFT 5.**

In bifidobacteria two *p-gtf* are present, galactosyl-transferase, *cspD*, and the undecaprenyl-phosphate sugar phosphotransferase, *rfb_P*, which appear to be located in almost all described EPS producing bifidobacteria. **DRAFT 5** described the computational approach used for designing and *in silico* validating three primer pairs for the amplification of the *rfb_P* bifidobacteria partial gene. Briefly, using bioinformatic tools, specific degenerated primer pairs were designed based on 15 aligned *rfb_P* partial gene sequences retrieved from the NCBI database. The choice of a good set of

primers represents a critical point still affecting both sensitivity and specificity of all PCR based protocols, therefore a Python script was *ad-hoc* written to support and to drive the primers selection. The script allowed us to rapidly cross and check 50 putative forward and 50 putative reverse oligonucleotides. Finally, the best three primer pairs were checked for specificity to target sequence using two different bioinformatic web servers.

4.4.3. RESULTS AND DISCUSSIONS

Results and Discussions from the *in silico* analysis are described in **DRAFT 5**.

In the first part of this research, a computational approach was used to develop a rapid and easy way for designing sets of degenerated primers targeting the priming glycosyltransferase *rfb_P* gene sequence. We aligned about 15 *rfb_P* partial gene sequences retrieved from the NCBI database; applying all methods described in the **DRAFT 5**, we were able to first select and then *in silico* validate the best sets of primers. Positive *in silico* results may suggest reliability of this primer pair in screening putative EPS-producing strains. To support these results and to verify the potential of our PCR based method, primers were *in silico* tested to verify the specificity of the primer pair for the target gene.

Positive *in silico* results may suggest reliability of this primer pair in screening putative EPSs-producing strains, but also the power of this study in defining a rapid, easy and economic way for degenerated primers design and for their *in silico* validation. However, an *in vivo* experiment is required, and was carried out in "Case of Study 4", in order to confirm or not the *in silico* results.

4.4.4. CONCLUSIONS

The choice of a good set of primers to detect the EPS producing strains, represents a critical point still affecting both sensitivity and specificity of all PCR based protocols. The obtained results suggest the power of this study in defining a rapid, easy and economic way for degenerated primers design and for their *in silico* validation. Additionally, this work suggests the potential of our primers as useful and rapid tool for the easily detection of the *rfb_P* gene in bifidobacteria strains, and to select candidate EPSs-producers. Nevertheless, *in vivo* analysis should be performed to support the hypothesis.

DRAFT 5

Michelini, S., Modesto, M., Michelini, T., Biavati, B. & Mattarelli, P. Computational approach for design and in silico validation of degenerate primer sets for the undecaprenyl-phosphate sugar phosphotransferase (rfb_P) partial gene amplification in *Bifidobacterium* spp.

Computational approach for design and in silico validation of degenerate primer sets for the undecaprenyl-phosphate sugar phosphotransferase (*rfb_P*) partial gene amplification in *Bifidobacterium* spp.

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Searching for exopolysaccharides (EPSs)-producing bacteria is a new challenge in putative probiotic strains selection. EPSs have several beneficial health-promoting effects due to their crucial roles in adhesion mechanisms, control of pathogens, maintaining survival/viability of microorganism during technology food process/storage, and contributing to rheological properties of fermented foods. The ability to produce EPSs has been recognized in several bifidobacterial strains but the mechanism of their biosynthesis is still hypothesized. The priming glycosyltransferase, *p-gtf*, should be the key enzyme in the catalyses of first step of EPS-units biosynthesis and the gene encoding for the *p-gtf*, undecaprenyl-phosphate sugar phosphotransferase (*rfb_P*) has been found in almost all producing bifidobacteria. Detecting the *rfb_P* via PCR should speed up the screening of putative producing microorganisms, avoiding the time-consuming EPSs extraction and quantification. For this study, a computational approach based on bioinformatic analyses was applied for designing proper degenerated primers sets allowing a partial amplification of *rfb_P*. Available bioinformatic tools and a Python script were used to support design, selection and for specificity checking of all degenerate primer sets. Positive results obtained from the *in silico* PCRs supported our approach and suggested the potentiality of three different degenerate primer sets in screening putative EPS-producing bifidobacteria.

Keywords: degenerate primer design, priming glycosyltransferase, bioinformatic, EPS, *Bifidobaterium*.

Introduction

Bifidobacteria are commonly inhabitants of the gastrointestinal tract of humans and animals (Mattarelli & Biavati, 2014). Their usage in foods and their claimed probiotic effects on host health may explain why they hold a great attraction for researchers. In recent years, there has been an increasing interest in probiotic bifidobacterial strains able to adhere to intestinal mucus. Furthermore, several studies described the production of exopolysaccharides (EPSs) in some strains belonging to the genus *Bifidobacterium* (Audy *et al.*, 2010; Hidalgo-Cantabrana *et al.*, 2014; Liu *et al.*, 2014; Ruas-Madiedo *et al.*, 2007; Shaun *et al.*, 2011). EPSs are carbohydrate polymers surrounding the cell of both Gram-positive and –negative bacteria, forming a capsule or a network link structure. Their production seems to be involved in host adhesion mechanism (Ruas-Madiedo *et al.*, 2012) and in ensuring persistence of the producing microorganisms in the gastrointestinal tract. Other important properties of EPSs are related to beneficial effects on human health (Ruas-Madiedo & de los Reyes-Gavilán, 2005), such as the control of bifidobacteria-host interaction, including the pathogens ability of commensal bacteria to remain immunologically silent and in turn provide pathogen protection (Fanning *et al.*, 2012). EPSs seem to be also important in maintaining survival and viability of microorganisms during the technology food process and storage (Grand *et al.*, 2003; Masco *et al.*, 2003; Stanton *et al.*, 1998), and in contributing to rheological properties of fermented food products (Audy *et al.*, 2010). Nowadays there has been also an increasing interest in their potential industrial applications (Madhuri & Prabhakar, 2014). This finding enforces the probiotic concept and provides new insights in putative probiotic strain selection.

Although the mechanism of EPSs biosynthesis in bifidobacteria is still unknown, the biosynthetic pathway described in lactic acid bacteria (LAB) has been proposed as hypothetical model subsequent to studies on sequence homology and on functional analysis of few genes (Hidalgo-Cantabrana *et al.*, 2014). In this supposed pathway, the first step in EPS-unit formation would be the transfer of a sugar-1-phosphate to the lipophilic carrier molecule anchored to the cell membrane. Formation of glycosidic bonds and transfer of new sugar moieties from donor nucleotide sugars to the initial monosaccharide of the EPS-unit would be catalysed by other *gtf*s. Once EPS-unit is finished, an export-polymerization process, mediated by flippase-like protein, would be necessary to move it from the cytoplasm to the extracellular face of the cytoplasmic membrane. Here, in the fourth step, a final assembling of the repeating units, takes place by a polymerase. For terminating chain elongation, other proteins responsible for chain-length determination would be involved, in turn influencing EPS molecular mass (Hidalgo-Cantabrana *et al.*, 2014).

In this model the priming glycosyltransferase, *p-gtf*, would be the key enzyme catalysing the first step of the EPS-units biosynthesis (Hidalgo-Cantabrana *et al.*, 2014). In most bifidobacteria strains the undecaprenyl-phosphate sugar phosphotransferase, *rfb_P*, was the putative *p-gtf* identified even if in some cases, the galactosyltransferase, *cspD* was also found (Hidalgo-Cantabrana *et al.*, 2014). These two genes showed a low amino acid homology to each other, but the intra-species homology of a single *p-gtf* is very high, maybe due to conserved domains involved in the interaction with the lipophilic carriers (Provencher *et al.*, 2003; Ruas-Madiedo *et al.*, 2007). Previous studies have confirmed the essential role of *p-gtf* gene in EPS production, as its inactivation alters or interrupts the biosynthetic pathway (van Kranenburg *et al.*, 1997; Low *et al.*, 1998; Stingele *et al.*, 1996). PCR amplification using specific primers may be a rapid tool for a quick screening of potential EPS-producing bifidobacteria strains. Nevertheless, sensitivity and specificity of a PCR reaction rely on selection of good primers, which in turns will depend upon bioinformatic analysis and bioinformatic tools. The aim of this work was to point out guidelines for proper design and validation of degenerate primer sets targeting bifidobacteria *rfb_P* partial gene. Primers were first designed using FAS-DPD program (Iserte *et al.*, 2013). Then, a selection of best primer sets was performed using a script written in Python, version 2.7.8 (https://www.python.org/) for this study, whereas their specificity was tested *in silico* using two web-servers, (i) *In-silico* PCR amplification, on http://insilico.ehu.es/PCR/ (San Millán *et al.*, 2013), and ii) MFE-primer 2.0, http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/index.cgi/#1 (Qu *et al.*, 2012). Results about specific amplification of *rfb_P* partial gene in bifidobacteria using the retrieved best primer pairs suggested the potential of this proposed strategy in designing degenerated primers sets. This study may be used as a model when proper design, selection and *in silico* validation of degenerated primers are required.

Materials and methods

1. Data Collection

In this work, primer pairs were designed on the available bifidobacterial *rfb_P* partial gene sequences listed by Hidalgo-Cantabrana et al. (2014) and retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). The complete list of sequences and related information are available in Table 1.

2. Degenerated Primer Design

Based on the assumption that genes with related function from different organisms show high sequence similarity, degenerate primers can be designed from sequences of homologues genes (Giegerich *et al.*, 1996). Indeed, designing a proper primer pair requires complete knowledge of the target sequence, in order to obtain specific DNA amplicon; however, when this information is missing, degenerated primers, representing a population of specific primers, should be used for amplifying all possible nucleotide sequences combinations coding for a given protein sequence (Iserte *et al.*, 2013).

Table 1. List of all strains used for the design of degenerated primer pairs. Accession numbers, presence of eps cluster, of *cspD* and *rfb_P* information were retrieved from Hidalgo-Cantabrana et al. (2014).

Species (references)	Strain	eps clusters	Genome	Accession Numbers
B. animalis* subsp. *lactis	DSM10140	+	complete	**CP001606**
B. animalis* subsp. *lactis	B1-04	+	complete	**CP001515**
B. animalis* subsp. *lactis	AD011	+	complete	**CP001213**
B. animalis* subsp. *lactis	BB-12	+	complete	**CP001853**
B. animalis* subsp. *lactis	V9	+	complete	**CP001892**
B. animalis* subsp. *lactis	BLC1	+	complete	**CP003039**
B. animalis* subsp. *lactis	CNCM I-2494	+	complete	**CP002915**
B. animalis* subsp. *lactis	Bi-07	+	complete	**CP003498**
B. animalis* subsp. *lactis	B420	+	complete	**CP003497**
B. breve	UCC2003	+	complete	**CP003039**
B. breve	JCM 7017	+	complete	**CP006712**
B. dentium	Bd1	+	complete	**CP001750**
B. longum* subsp. *infantis	ATCC15697	+	complete	**CP001095**
B. longum* subsp. *longum	NCC2705	+	complete	**AE014295**
B. longum* subsp. *longum	F8	+	complete	**FP929034**
B. longum* subsp. *longum	KACC 91563	+	complete	**CP002794**

For primers finding, FAS-DPD 1.1.2, a program developed and implemented by Iserte et al. (2013) for solving problems with degenerate primer design (DPD) on known/unknown members of gene families or organism families, have been employed. Since program starts with a DNA sequence alignment as input file, the retrieved *rfp_B* sequences were first aligned in CLC_Sequence Viewer version 7.5, for Mac OS (CLC, Inc., Aarhus, Denmark) using Clustal Omega (Sievers *et al.*, 2011) and default parameters for both pairwise and multiple alignments. Then, resulting aligned sequences (Supplementary Material S1) were analysed for finding the most conserved region of the gene and were cut in the less conserved part to obtain sequences of about the same length; this alignment was than run in FAS-DPD program. Length of primers was set at 20 bp and default parameters for identification of 50 potential degenerate forward primers, on the direct strand, and 50 reverse primers, on the complementary strand, were used. Two different ranking lists of oligonucleotides, for forward and reverse respectively, with information related to score threshold were obtained as output files. The best scoring primers may not be the less degenerated, but take into account a biological restraint no considered in other methods, indeed the value contains degeneracy but weighted by its proximity to the 3' end of the primer minimizing the degeneracy at that end while allowing more freedom in the remaining positions (Iserte *et al.*, 2013).

3. Degenerated Primer Pairs Selection

Screening of putative degenerated primers from output files and subsequent selection of the best sets were performed using a script written in Python, version 2.7.8 (https://www.python.org/). The script (Supplementary Material S2) directly works on the output files obtained with FAS-DPD and allows the user for filtering parameters, such as scores threshold, minimum and maximum length of amplicon, minimum, maximum and optimal mealting temperature (Tm), G+C content (GC%), sodium salt concentration [Na^+] and number of nucleotides that may give self- and cross dimerization. The Tm may be calculated using either the Marmur and Doty equation (1962) adjusted for [Na^+] if primers are shorter then 14 bp, or the equation of Wallace et al. (1979), adjusted for [Na^+], when they are longer then 14 bp. Firstly, forward and reverse primers with a given score threshold, in our case ≥ 0.95 and ≥ 0.93 respectively, were selected for the further processing steps. Two different scores were chosen in order to compare a similar number of forwards and reverses (about 20), indeed low values were registered in the reverse oligonucleotides. Each obtained forward and reverse primer were paired and only those unable to produce amplicon of size ranging from 135 to 250 bp were left off all subsequent analysis. A further selection was also carried out to check for self

– dimerization on degenerated oligonucleotides. For resulting putative primers, information about GC% and Tm were calculated. Tm for degenerated oligonucleotides with more than 14 bp was used as option. Only primers showing a GC% ranging from 40 to 50% and a Tm between 45 and 70°C were further screened. Promising primer pairs were selected considering the difference of melting temperature between each possible primer pair, $\Delta Tm \leq 6$, and a length of amplicon ranging from 135 to 250 bp. Relative errors, standardized on the optimal values for GC% (46%) and Tm, (62°C) were calculated for each primer pair. The mean value of relative errors was used as score average for ranking putative primer pairs. The absence of cross – dimerization between primers of each pair was also checked. The resulting primer pairs and relatives scores were loaded in a comma - separated values (CSV) format file.

The best primer pairs resulted from CSV needed a manual editing to remove degenerate Gs or Cs at the 3' - end, which could promote mispriming at G or C - rich sequences (because of stability of annealing) (Chen *et al.*, 2002), or to add/cut some nucleotides at the ends if it would be preferred. This step was not inserted in the script in order to give users the possibility to improve themselves specificity of the primers pairs, if it would be required; a subsequent analysis should be performed for assuring no changes in primer parameters resulting from these modifications. For this aim, the web-tool Multiple Primers Analyzer (ThermoScientific, http://www.thermoscientificbio.com) was employed for information about GC content, annealing temperature, hairpin formation and cross - dimerization.

4. Validation of the best primer pair

The validation of the best putative primer pair was performed *in silico* using two different web-server tools, which allow degenerate primers as input, i) *In silico* PCR amplification, on http://insilico.ehu.es/PCR/ (San Millán *et al.*, 2013), and ii) MFE-primer 2.0, http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/index.cgi/#1 (Qu *et al.*, 2012).

i) Employing the *In silico* PCR amplification program, a simulation of PCR using the best primer pair was performed against the available bifidobacterial genomes and draft genome databases, updated by NCBI. A second amplification was also carried out against those genomes, given as user's input since missing in the available database, which were analysed by Hidalgo-Cantabrana et al. (2014) and listed in Table 1. Finally, similarity sequences of the resulting amplicons were obtained using the BLAST search tool against the NCBI database http://blast.ncbi.nlm.nih.gov/.

ii) The PCR primers specificity was also checked using the server MFE-primer 2.0, against the currently updated bacterial genomes and bifidobacterial draft genomes in NCBI database. Primer pair coverage (PPC%) of 70% was set as cut-off for defining the efficiency of the primer pair in binding the DNA template. This server employs a k-*mer* index algorithm for accelerating the search process for primer binding sites and it uses thermodynamics to evaluate binding stability between each primer and its related DNA template (Qu *et al.*, 2012). It was preferred to the Primer - BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) as it supports degenerate primers analysis and it employs a thermodynamic approach to predict reasonable unintended primer binding sites (Qu *et al.*, 2012).

Results and Discussion

Based on data of *rfb_P* gene distribution in bifidobacteria species retrieved from Hidalgo-Cantabrana et al. (2014) and from the NCBI database, after an earlier selection for eliminating too short or too gap-opened sequences, a total of 15 partial *rfb*_P gene sequences (underlined in Table 2), were aligned with Clustal Omega in CLC_Sequence Viewer, which not allow tu set user's parameters. Sequences were manually edited avoiding initial and final gaps to obtain aligned sequences of about 1450 bp (Fig. 1).

FAS-DPD program was then used for designing 50 putative degenerate forward and reverse primers on the given alignment. Lists of the best primers (score > 0.95 and >0.93 for forwards and reverses, respectively) and their parameters were showed in Table 2.

A Degenerated Primer Pairs Selection (DPPS) python script was specially written for the selection of oligonucleotides and the detection of the best primer pairs of this study. For retrieving the best set, after a base - score selection, single oligonucleotides and each possible primers combination were filtered and ranked for amplicon length, GC%, Tm, self - and cross - dimer

formation, hairpin estimation and ΔTm. A manual editing of oligonucleotides would be needed to remove degenerate bases at the 3'-end, increasing their specificity.

Table 2. FAS-DPD forward and reverse primer lists with parameters.

Primer Name	Forward Primers (5'-3')	Score (≥0.95)	Start	End	Direct Strand
rfb_P_for1	TGTACAARTTCCGTTCCATG	0.989	1037	1056	TRUE
rfb_P_for2	ATGTACAARTTCCGTTCCAT	0.987	1036	1055	TRUE
rfb_P_for3	BATGTACAARTTCCGTTCCA	0.983	1035	1054	TRUE
rfb_P_for4	MBATGTACAARTTCCGTTCC	0.978	1034	1053	TRUE
rfb_P_for5	MMBATGTACAARTTCCGTTC	0.972	1033	1052	TRUE
rfb_P_for6	TTCAAGVTSAAGGACGATCC	0.971	1123	1142	TRUE
rfb_P_for7	HTTCAAGVTSAAGGACGATC	0.965	1122	1141	TRUE
rfb_P_for8	YMMBATGTACAARTTCCGTT	0.964	1032	1051	TRUE
rfb_P_for9	AYGTGYTSAARGGCGATATG	0.961	1208	1227	TRUE
rfb_P_for10	YHTTCAAGVTSAAGGACGAT	0.958	1121	1140	TRUE
rfb_P_for11	TYMMBATGTACAARTTCCGT	0.956	1031	1050	TRUE
rfb_P_for12	AAYGTGYTSAARGGCGATAT	0.956	1207	1226	TRUE
rfb_P_for13	AYHTTCAAGVTSAAGGACGA	0.950	1120	1139	TRUE

Primer Name	Reverse Sequence (5'-3')	Score (≥0.93)	Start	End	Direct Strand
rfb_P_rev1	BWRCGYCCGGARATYTGCCA	0.943	1346	1327	FALSE
rfb_P_rev2	MCACHGTCTTSADCAGAATC	0.942	1441	1422	FALSE
rfb_P_rev3	CCMACCAWRSWCATATCGCC	0.941	1238	1219	FALSE
rfb_P_rev4	TTSARCACRTTRARGAACTG	0.938	1217	1198	FALSE
rfb_P_rev5	ACCAWRSWCATATCGCCYTT	0.937	1235	1216	FALSE
rfb_P_rev6	ATRTCSCCSGTRATMGACCA	0.936	1418	1399	FALSE
rfb_P_rev7	ARCACRTTRARGAACTGMGG	0.934	1214	1195	FALSE
rfb_P_rev8	MMCACHGTCTTSADCAGAAT	0.933	1442	1423	FALSE
rfb_P_rev9	SWCATATCGCCYTTSARCAC	0.933	1229	1210	FALSE
rfb_P_rev10	RBWRCGYCCGGARATYTGCC	0.932	1347	1328	FALSE
rfb_P_rev11	VCCMACCAWRSWCATATCGC	0.932	1239	1220	FALSE

Table 3. Output from the DPPS python script; primers name were added and the best three primer pairs are in bold.

Forward		**Reverse**			
Primer name	**Sequences (5'-3')**	**Primer name**	**Sequences (5'-3')**	**Score_err**	**Ampl_length**
rfb_P_for4	**MBATGTACAARTTCCGCC**	**rfb_P_rev7**	**ARCACRTTRARGAACTGMGG**	**0.052**	**181.0**
rfb_P_for1	**TGTACAARTTCCGTTCCATG**	**rfb_P_rev7**	**ARCACRTTRARGAACTGMGG**	**0.054**	**178.0**
rfb_P_for9	**AYGTGYTSAARGGCGATATG**	**rfb_P_rev2**	**MCACHGTCTTSADCAGAATC**	**0.058**	**234.0**
rfb_P_for3	BATGTACAARTTCCGTTCCA	rfb_P_rev7	ARCACRTTRARGAACTGMGG	0.067	180.0
rfb_P_for5	MMBATGTACAARTTCCGTTC	rfb_P_rev7	ARCACRTTRARGAACTGMGG	0.072	182.0
rfb_P_for4	MBATGTACAARTTCCGTTCC	rfb_P_rev5	ACCAWRSWCATATCGCCYTT	0.076	202.0
rfb_P_for12	AAYGTGYTSAARGGCGATAT	rfb_P_rev2	MCACHGTCTTSADCAGAATC	0.078	235.0
rfb_P_for4	MBATGTACAARTTCCGTTCC	rfb_P_rev9	SWCATATCGCCYTTSARCAC	0.0782	196.0
rfb_P_for1	TGTACAARTTCCGTTCCATG	rfb_P_rev5	ACCAWRSWCATATCGCCYTT	0.078	199.0
rfb_P_for9	AYGTGYTSAARGGCGATATG	rfb_P_rev8	MMCACHGTCTTSADCAGAAT	0.079	235.0
rfb_P_for1	TGTACAARTTCCGTTCCATG	rfb_P_rev9	SWCATATCGCCYTTSARCAC	0.080	193.0
rfb_P_for4	MBATGTACAARTTCCGTTCC	rfb_P_rev4	TTSARCACRTTRARGAACTG	0.087	184.0
rfb_P_for1	TGTACAARTTCCGTTCCATG	rfb_P_rev4	TTSARCACRTTRARGAACTG	0.089	181.0
rfb_P_for3	BATGTACAARTTCCGTTCCA	rfb_P_rev5	ACCAWRSWCATATCGCCYTT	0.092	201.0
rfb_P_for3	BATGTACAARTTCCGTTCCA	rfb_P_rev9	SWCATATCGCCYTTSARCAC	0.094	195.0
rfb_P_for5	MMBATGTACAARTTCCGTTC	rfb_P_rev5	ACCAWRSWCATATCGCCYTT	0.097	203.0
rfb_P_for5	MMBATGTACAARTTCCGTTC	rfb_P_rev9	SWCATATCGCCYTTSARCAC	0.099	197.0
rfb_P_for12	AAYGTGYTSAARGGCGATAT	rfb_P_rev8	MMCACHGTCTTSADCAGAAT	0.099	236.0
rfb_P_for3	BATGTACAARTTCCGTTCCA	rfb_P_rev4	TTSARCACRTTRARGAACTG	0.102	183.0
rfb_P_for5	MMBATGTACAARTTCCGTTC	rfb_P_rev4	TTSARCACRTTRARGAACTG	0.107	185.0

Resulting best primer pair sets were showed in Table 3. The specificity of the selected primer pairs was tested using two web - servers, (i) *In Silico* PCR amplification and (ii) the MFE-primer 2.0. Results from the *in silico* PCR were summarised in Table 3 whereas amplicons obtained from the two programs for each tested primer pair are available as Supplementary Material S3-S8. Both servers resulted able to identify fragments belonging to *Bifidobacterium* spp of about 181, 178 and 234 bp, respectively by using the first three primer sets. Every one of which also showed high binding stability with DNA template among whole bacterial genome. BLAST of amplicons matched with bifidobacterial gene involved in the biosynthesis of exopolysaccharides and belonging to the family of the polyprenyl glycosyl-phosphotransferase. All bifidobacterial *rfb_P* gene sequences retrieved from the NCBI database and those obtained as amplicons with *in silico* PCR, were aligned for phylogenetic tree reconstruction (Fig. 2).

i) *In Silico* PCR amplification web-server was used to test primers specificity against *Bifidobacterium* and against other user's genomes, thus screening a total of 37 strains. All the three checked sets of primers, showed a high specificity (about 96.2%) when amplifying their target region whether from strains harbouring the priming *rfb_P* (Table 3) or from two strains of *B. animals* subsp. *lactis* and *B. bifidum* S17, never screened before by Hidalgo-Cantabrana et al. (2015). No amplicon resulted from those strains harbouring only the *cspD* gene, such as *B. adolescents* ATCC 15703, *B. animalis* subsp. *lactis* ATCC 27673, *B. animals* subsp. *animals* ATCC 25527, *B. asteroides* PRL2011, *B. bifidum* PRL2010 and *B. bifidum* BNG4, *B. dentium* Bd1, *B. longum* subsp. *longum* DJ010A, *B. longum* subsp. *longum* JDM301, *B. longum* subsp. *longum* JCM 1217 and *B. longum* subsp. *longum* BBMN68. An *in vivo* analysis is required to confirm all these results obtained. However, no amplicon resulted from *B. thermophilum* RBL67 even if this strain was found to harbour the target gene. This result was in agree with the recent study of Hidalgo-Cantabrana et al. (2015) and may be explained with the high genetic variability of the gene in this strain. The analysis against *Bifidobacterium* spp. draft genomes, listed in Table 4, resulted in no amplification maybe due to the failure in allowing mismatch between primers and sequences for this kind of analysis.

ii) The first three sets of primers were also used to amplify *rfb_P* gene from the Bacterial genome database available in MFE-primer 2.0 tool. Three amplicons of about 181, 178 and 234 bp size were obtained and the BLAST ascribed each one of them to the *rfb_P* gene, supporting the specificity of our designed primers. The MFE-primer 2.0 program also suggested higher power efficiency (100.0%) for selected oligonucleotides in amplifying their target from all *Bifidobacterium* strains, which harbour the gene. However, it also to be underlined that only strains harbouring this gene were found in the available database. Furthermore, when target amplification was tested against draft *Bifidobacterium* genomes (Table 4), the sets of primers gave different response from each other. All of them resulted able to detect *B. animalis* subsp. *lactis* BS01_05, *B. breve* CECT 7263 and *B. breve* DSM 20213, whereas only the first set amplified also from *B. longum* subsp. *infantis* CUCG 52486, *B. longum* subsp. *longum* 2-2B and *B. longum* subsp. *longum* 35B. The BLAST results confirmed that amplicons were related to exopolysaccharide biosynthesis polyprenyl glycosyl-phosphotransferase proteins family.

A suitable specificity of all three sets of primers pairs in amplifying *rfb_P* fragments resulted from both *in silico* PCR, mainly using the *In silico* PCR tool, which also allows to test user's sequences. Neither aspecific bands nor aspecific amplification of *cspD,* the other *p-gtf* appear to be obtained, though being present in some bifidobacterial genomes like *B. animalis* subsp. *animalis* ATCC 2552, *B. longum* subsp. *longum* JCM 1217 and *B. longum* subsp. *longum* BBMN68. Furthermore, the first set of primers may be considered more suitable than the other two, as it amplified also from draft genomes of more strains, *B. animalis* subsp. *lactis* BS01_05 (AHGW01000005)*, B. breve* CECT 7263 (NZ_AFVV01000001) and DSM 20213 (NZ_JDUD00000000), *B. longum subsp. longum* 2-2B (NZ_AJTJ00000000) and 35B (NZ_AJTI00000000), but also *B. longum subsp. infantis* CCUG 52486 (NZ_ABQQ00000000). Results could support that the two *p-gtf*, *rfb_P and cspD* shared a low homology to each other and this finding correlated with the results of Hidalgo-Cantabrana et al. (2014). Positive *in silico* amplifications suggested the potential of this work as guideline for designing and selecting specific degenerated primer pairs. However, to confirm the effective specificity of our oligonucleotides, an *in vivo* analysis is needed and will be carried out.

Conclusions

A computational approach was used to develop a rapid and easy way for designing sets of degenerated primers targeting the priming glycosyltransferase *rfb_P* gene sequence, harboured in many strains of *Bifidobacterium*; the gene was found to encode

for the key enzyme involved in the bifidobacterial EPSs biosynthesis (Hidalgo-Cantabrana et al. 2014). Recently, studies on the possibility to screen EPS-producing bifidobacteria via PCR, have gained increased interest and searching for the presence of the priming glycosyltransferase genes seems to be the right choice (Provencher et al. 2003; Hidalgo-Cantabrana et al. 2014; Hidalgo-Cantabrana et al. 2015). However the choice of a good set of primers represents a critical point still affecting both sensitivity and specificity of all PCR based protocols. For this work, we aligned about 15 *rfb_P* partial gene sequences retrieved from the NCBI database, and applying all methods described above, we were able to first selecting and then in silico validating the best sets of primers. Positive in silico results may suggest reliability of this primer pair in screening putative EPS-producing strains, but also the power of this study in defining a rapid, easy and economic way for degenerated primers design and for their in silico validation. However, an in vivo experiment is required, and will be carried out, for confirming or not the in silico results.

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Table 4. List of strains used in this study, accession numbers, cspD and rfb_P information, and in silico PCR results; draft genomes are retrieved from NCBI. Rfb_P gene. Underlined strains refer to sequences that were used for the degenerate primer designed, whole genomes of the other strains were used for the in silico PCR. NO, if there was not amplification, YES, if there was. nd=not in the database.

Species	**Strain**	**Genome Acc. Number**	***p-gtf***		***in silico* PCR**			**MFE-primer 2.0**		
			cspD	***rfb_P***	I Primer Pairs 181	II Primer Pairs 178	III Primer Pairs 234	I Primer Pairs 181	II Primer Pairs 178	III Primer Pairs 234
B. adolescentis	ATCC15703	AP009256	BAD_1389	-	NO	NO	NO	NO	NO	NO
B. animalis* subsp. *animalis	ATCC 25527	CP002567	BANAN_06765	-	NO	NO	NO	NO	NO	NO
B. animalis* subsp. *lactis	DSM10140	CP001606	Balat_1392	Balat_1371	YES	YES	YES	YES	YES	YES
	B1-04	CP001515	Balac_1392	Balac_1371	YES	YES	YES	YES	YES	YES
	AD011	CP001213	BLA_0595	BLA_0576	YES	YES	YES	YES	YES	YES
	BB-12	CP001853	BIF_00944	BIF_00983	YES	YES	YES	YES	YES	YES
	V9	CP001892	BalV_1349	BalV_1328	YES	YES	YES	YES	YES	YES
	BLC1	CP003039	BLC1_1349	BLC1_1328	YES	YES	YES	YES	YES	YES
	CNCM I-2494	CP002915	BALAC2494_01344	BALAC2494_01362	YES	YES	YES	YES	YES	YES
	Bi-07	CP003498	W91_1429	W91_1409	YES	YES	YES	YES	YES	YES
	B420	CP003497	W7Y_1394	W7Y_1374	YES	YES	YES	YES	YES	YES
	Bl12	NC_021593	nd	Bl12_1287	YES	YES	YES	nd	nd	nd
(Liu *et al.*, 2014)	RH	CP007755	-	*rfb_P*	YES	YES	YES	nd	nd	nd
(Loquasto *et al.*, 2013)	ATCC 27673	CP003941	-	-	NO	NO	NO	nd	nd	nd
B. asteroides	PRL2011	CP003325	BAST_1667	-	NO	NO	NO	nd	nd	nd
B. bifidum	PRL2010	CP001840	-	-	NO	NO	NO	NO	NO	NO
	S17	CP002220	-	BBIF_0393	YES	YES	YES	YES	YES	YES
	BGN4	CP001361	-	-	NO	NO	NO	NO	NO	NO
B.breve	ACS-071-V-Sch8b	CP002743	-	HMPREF9228_0447	YES	YES	YES	YES	YES	YES
	UCC2003	CP000303	-	Bbr_0430	YES	YES	YES	nd	nd	nd
	NCFB 2258	CP006714	nd	B2258_0400	YES	YES	YES	nd	nd	nd
	689b	CP006715	nd	B689b_0425	YES	YES	YES	nd	nd	nd

	S27	CP006716	nd	BS27_0430	YES	YES	YES	nd	nd	nd
	12L	CP006711	nd	B12L_0366	YES	YES	YES	nd	nd	nd
	JCM 7019	CP006713	-	B7019_0391	YES	YES	YES	nd	nd	nd
	JCM 7017	CP006712	-	B7017_0382	YES	YES	YES	nd	nd	nd
B. dentium	Bd1	CP001750	BDP_1855 BDP_1857	-	NO	NO	NO	NO	NO	NO
B. longum* subsp. *infantis	ATCC15697	CP001095	-	Blon_2114	YES	YES	YES	YES	YES	YES
	157F	AP010890	-	BLIF_0362	YES	YES	YES	YES	YES	YES
B. longum* subsp. *longum	NCC2705	AE014295	BL0237	BL0249	YES	YES	YES	YES	YES	YES
	DJ010A	CP000605	-	-	NO	NO	NO	NO	NO	NO
	F8	FP929034	-	BIL_15040	YES	YES	YES	nd	nd	nd
	JDM301	CP002010	-	-	NO	NO	NO	NO	NO	NO
	JCM 1217	AP010888	BLLJ_0364	-	NO	NO	NO	NO	NO	NO
	KACC 91563	CP002794	-	BLNIAS_02272	YES	YES	YES	YES	YES	YES
	BBMN68	CP002286	BBMN68_1012	-	NO	NO	NO	NO	NO	NO
B. thermophilum	RBL67	CP004346	-	D805_0348	NO	NO	NO	nd	nd	nd

Species	**Strain**	**Draft Genome Acc. Number**	***p-gtf***		**MFE-primer 2.0**			***in silico* PCR**		
			cspD	***rfb_P***	I Primer Pairs 181	II Primer Pairs 178	III Primer Pairs 234	I Primer Pairs 181	II Primer Pairs 178	III Primer Pairs 234
B. actinocoloniforme	12-1-47BFAA	NZ_ADCN00000000	-	-	NO	NO	NO	NO	NO	NO
B. adolescentis	L2-32	NZ_AAXD00000000	-	-	NO	NO	NO	NO	NO	NO
B. animalis* subsp. *lactis	HN019	NZ_ABOT00000000	-	-	NO	NO	NO	NO	NO	NO
B. animalis* subsp. *lactis	BS01_05	AHGW01000005	-	-	NO	NO	NO	YES	YES	YES
B. angulatum	DSM 20098	NZ_JDTY00000000	-	-	NO	NO	NO	NO	NO	NO
B. bifidum	LMG 13195	NZ_AMPL00000000	-	-	NO	NO	NO	NO	NO	NO
	IPLA 20015	NZ_AMPM00000000	-	-	NO	NO	NO	NO	NO	NO
	NCIMB 41171	NZ_ABQP00000000	-	-	NO	NO	NO	NO	NO	NO
B. breve	CECT 7263	NZ_AFVV01000001	-	-	NO	NO	NO	YES	YES	YES

	DSM 20213	NZ_JDUD00000000	-	-	NO	NO	NO	YES	YES	YES
B. catenulatum	DSM 16992	NZ_JDTP00000000	-	-	NO	NO	NO	NO	NO	NO
B. dentium	DSM 27678	NZ_ABIX00000000	-	-	NO	NO	NO	NO	NO	NO
	DSM 27679	NZ_AEEQ00000000	-	-	NO	NO	NO	NO	NO	NO
	JCVIHMP022	NZ_AEHJ00000000	-	-	NO	NO	NO	NO	NO	NO
B. gallicum	DSM 20093	NZ_JDTW00000000	-	-	NO	NO	NO	NO	NO	NO
B. longum subsp. infantis	CCUG 52486	NZ_ABQQ00000000	-	-	NO	NO	NO	YES	NO	NO
	ATCC 55813	NZ_ACHI00000000	-	-	NO	NO	NO	NO	NO	NO
B. longum subsp. longum	44B	NZ_AJTM00000000	-	-	NO	NO	NO	NO	NO	NO
	1-6B	NZ_AJTF00000000	-	-	NO	NO	NO	NO	NO	NO
	2-2B	NZ_AJTJ00000000	-	-	NO	NO	NO	YES	NO	NO
	35B	NZ_AJTI00000000	-	-	NO	NO	NO	YES	NO	NO
B. longum subsp. longum	DJO10A	NZ_AABM00000000	-	-	NO	NO	NO	NO	NO	NO
B. minimum	DSM 20102	NZ_ATXM00000000	-	-	NO	NO	NO	NO	NO	NO
B. pseudocatenulatum	DSM 20438	NZ_JDUK00000000	-	-	NO	NO	NO	NO	NO	NO

4.5. CASE OF STUDY 3: EPSs PRODUCTION BY SIX *Bifidobacterium aesculapii* STRAINS IN DIFFERENT SUBSTRATES

Fermented milks, obtained from the spontaneous fermentation of milk by microorganism, have been historically used by nomadic populations of the Arabic peninsula, the Caucasus and the Anatolia, which based their nutrition on milks and its derived products (Del Bono and Stefani., 1997). Indeed, the acidification of milk increases the shelf life and beneficial properties of the final product (Vizzardi and Maffeis, 1990).

International Dairy Federation defined a fermented milk product as "the milk product prepared from skimmed milk or not with specific cultures. The microflora is kept alive until sale to the consumers and may not contain any pathogenic germs" (Panesar, 2011). There are heterogeneous fermented milks determined by environmental factors and by microbial population, mainly composed by lactic acid bacteria, LAB, and yeasts, involved in the fermentation process. Fermented milks could be classified in different ways, based on the characteristics of the main microorganisms (thermophilic, mesophilic or alcoholic acid mikes) (Ottogalli and Testolin, 1991) or the sensorial characteristics (acid, acid- alcoholic, scarcely acid, scarcely acid added with mesophilic LAB, scarcely acid added with probiotics). The main fermented milks products are yogurt, acidophilus milk, mayzum, buttermilk, kefir, kumis and leben. These dairy foods are included in the list of the "functional foods" when added with probiotics (Leroy and De Vuyst, 2014). Even though fermented milks added with probiotics enhance the human health, their sensorial characteristics play a crucial role in the acceptance by consumers of the products (Gardini et al., 1999)

Probiotics, isolated from both human intestinal tract and foods, are widely used in the production of fermented milks or cheeses due to their functional properties (Law and Hansen, 1997). Strains belonging to *Lactobacillus* spp. and *Bifidobacterium* spp. are the main used probiotic bacteria in the fermented milk products. However, bifidobacteria are not able to fully complete their probiotic functionalities when added to milk based products because negative factors, such as pH and oxygen may influence their growth (Prasanna et al., 2014). For example, in the gastrointestinal tract of humans, compared to *Lactobacillus acidophilus,* bifidobacteria are more affected by the stomach conditions, such as pH and bile salt concentration (Ferdousi et al., 2013). Many factors, such as process parameters, packaging and storage, could affect the survival, the viability and the activity of probiotics. For example, in order to overcome vitality losses, it is commonly to prefer *Streptococcus thermophilus* as starter instead *Lactobacillus delbrueckii* subsp. *bulgaricus*, which increase the acidity of the product during the fermentation (Mortazavi et al., 2005; Tamime et al., 2008).

Among bifidobacteria, the literature identified Bifidobacterium longum and Bifidobacterium pseudolongum strains as the most resistance to acidity and bile salt (Lankaputhra & Shah, 1995). B. longum spp., B. infantis and B. breve are the specie commonly used in the production of yoghurts (Shah, N.P. & Lankaputhra, 1997; Lankaputhra & Shah, 1995). In particular B. longum spp. exhibits high vitality in presence of other microorganisms used as starters for yogurth (Dawson-Hughes et al., 1990). Effective incorporation of probiotics into fermented products, requires that probiotics bacteria maintain their viability without producing off-flavors or adversely altering the sensory characteristics, either fermentation parameters or shelf-life. For technological purpose the better choice is a probiotic strain able to growth in the dairy matrix. An important point to be considered is that fermented milks obtained by the use of only probiotic strains, such as Bifidobacterium spp. or L acidophilus are generally affected by bad sensorial quality, textural deficiency and lack of flavor (Marshall, 2009). The main compound characterizing the aroma of yogurt is the acetaldehyde, but also ethanol, acetone, diacetyl and 2-butanone are often detected (Kneifel & Peinemann, 1992).

Incorporation of EPS-producing LAB in various fermented products, such as fermented milk, cheese and fermented beverage, has become a recent trend. The EPS-producing LAB strains have increasingly been used as functional starter cultures for manufacturing fermented products due to their capability of improving rheology, texture and mouthfeel, and reducing thermal and physical shock and syneresis of the products. In recent years, different EPS producing species of LAB have been used in fermented milk to prevent syneresis and to replace stabilizers. Streptococci, lactobacilli, lactococci and bifidobacteria are some EPS-producing bacterial species which have been successfully used to produce fermented milk with varying improvement of physicochemical and biological properties (Wang et al., 2015). The EPSs can modify the flow characteristics of fluids, stabilize suspensions, flocculate particles, encapsulate materials and produce emulsions (Charchoghlyan & Park, 2013). Therefore, incorporation of EPS-producing LAB in various soybean-based products have become a recent trend (Mende et al., 2013).

The EPSs produced by LAB have been shown to improve the texture (Welman & Maddox, 2003) and have commercially been used as a fat substitute in low-fat products and for obtaining an increased mouth thickness (Prasanna et al., 2012).

EPS production from bifidobacteria is currently well documented (Salazar et al., 2015, 2008; Ruas-Madiedo et al., 2007; Welman & Maddox, 2003; Hidalgo-Cantabrana et al., 2014; Ruas-Madiedo et al., 2002), and a sugar source modulation on the EPSs biosynthesis in B. longum subsp. longum CRC 002 has been demonstrated with the work of Audy et al. (2010). However, to date, there is little information on the use of EPS-producing Bifidobacterium strains as functional starters in low-fat fermented milk products (Prasanna et al., 2012).

4.5.1. AIM OF THIS WORK

This part of the work was aimed at quantifying EPSs production in seven strains of the novel species *Bifidobacterium aesculapii*. All strains were grown in TPY broth with different carbon sources (glucose or lactose), in low fat and in soybean milk. EPSs were extracted and quantified from all matrices. The aromatic compounds profiles and the texture of fermented milks were also analysed.

4.5.2. MATERIALS AND METHODS

The *B. aesculapii* strains used in this study are listed in Table 10, *Bifidobacterium longum* subsp. *infantis* ATCC 15697 and *Bifidobacterium saguini* DSM 23967^T were also included as controls. All strains were revitalized from freeze-dried, in TPY medium and incubated anaerobically at 37°C for 24 hours.

Species	Strain	Collection Number
B. aesculapii	MRM 3.1	DSM 26737^T
B. aesculapii	MRM 4.2	DSM 26738
B. aesculapii	MRM 4.6	-
B. aesculapii	MRM 4.7	-
B. aesculapii	MRM 4.8	-
B. aesculapii	MRM 5.13	-
B. aesculapii	MRM 8.7	-
B. longum subsp. *infantis*	RE 06	ATCC 15697
B. saguini	-	DSM 23967^T

Table 10. List of strains tested.

a) EPSs production by strains in TPY with different carbon sources

To verify relatedness between EPSs production and presence of different carbon sources in the growing medium, 10% of an overnight culture of each strain was inoculated and cultured three times anaerobically at 37°C for 18 hours in TPY modified by addition of 1.5 (control) and 2% of glucose, 1,5% and 2% of lactose. All the analyses were performed in triplicate. At the end of incubation time and before EPS quantification, samples were removed for viable counts.). Viable counts (CFU/mL) were determined by plating 10-fold serial dilutions in TPY agar. Plates were incubated in anaerobic condition at 37°C for 24-48 hours.

All strains were also tested for their ability to grow in whole or low fat milk (Elisyr, Parmalat) or in soy milk, (SoyaDrink, Valsoia) thus assessing their contribution to the viscosity of the fermented milk. Ingredients and nutritional values of commercial milks are showed in Table 11 and Table 12.

Ingredients	
Low-fat milk (UHT)	**Soybean milk**
Milk (1% of fat)	Water
Zinc sulphate	Soybeans (6.8%)
Sodium selenite	Brown sugar
Vitamins: B1, B3, B5, B6, B9, B12, D, E	Tricalcium phosphate
	Sea salt
	Stabilizer: gellan
	Aroma
	Vitamins: B2, B12, D

Table 11. Ingredients contained in 100 ml of law-fat milk UHT, Elysi Parmalat, and soybean milk, SoyaDrink Valsoia.

Nutritional values (100 ml)			
Low-fat milk (UHT)		**Soybean milk**	
Energy	175 kj, 41 kcal	Energy	158 kJ, 38 kcal
Fats	1.0 g	Fat	1.7 g
Carbohydrates	4.9 g	of which satured	0.3 g
Proteins	3.2 g	Carbohydrates	2.5 g
Calcium	120 mg	of which sugar	2.1 g
Zinc	1.5 mg	Fibres	0.2 g
Salt	0.13 g	Proteins	3 g
Selenium	8.25 µg	Salt	0.1 g
Vitamins:		Calcium	120 mg
D	0.75 µg		
E	1.8 mg		
B1	0.17 mg		
B6	0.21 mg		
B12	0.4 µg		
B9	30 µg		
B5	0.9 mg		

Table 12. Nutritional values of 100 ml of law-fat milk UHT, Elysi Parmalat, and soybean milk, SoyaDrink Valsoia.

After revitalizing and sub-cultivation in TPY broth, cells of each strain were collected by centrifugation (6000 rpm for 20 min) and washed twice with phosphate buffer saline (PBS). Pellets from 60 ml of overnight cultures were concentrated by suspending in 15 ml of soybean milk and skim milk (Oxoid) added with 2.5% of yeast extract, which represented the inoculums. 5ml of each

preparation was used to inoculate 45 ml of low-fat or soybean milk and incubated aerobically at 37°C for 24-48 hours. All the tests were performed in triplicate.

Samples were collected for determinations of viable counts before and after fermentation.

b) EPSs extraction and quantification

Extraction of EPSs and quantification were performed according to the method by (Dubois et al., 1956). Briefly, after adjustment to pH 7, 100 µl of Flavourzime (10%) were added to each sample and vortexed for 15 sec. before incubation at 50°C for 4 hours by gently stirring. 500 µl of each sample were transferred in a 10 ml plastic tube containing 2.9 ml of ultra pure water and 7 ml of cold absolute ethanol and finally incubated overnight at 4°C. Pellets were recovered by centrifugation at 27000 g for 40 min at 4°C and air dried for 10 min. Further 7 ml of cold absolute ethanol were added to each sample and incubated overnight at 4°C. Samples were centrifuged at 27000 g for 40 min at 4°C and air dried for 10 min; pellets were resuspended in 1 ml of ultra pure water. An aliquot of 7% of a phenol solution (80% in water) were added to each sample and vortexed for 15 sec; tubes were transferred on ice before adding 5 ml of sulphuric acid (97%). After 30 min, 2 ml of each sample were used for the optical density (OD) measurement at 485 nm by spectrophotometer. Quantifications of EPSs produced by each strain at tested conditions were retrieved by comparison with a standard curve. The standard curve was built based on the OD obtained from water solutions at different concentration of glucose (400 ppm, 200 ppm, 100 ppm, 75 ppm, 50 ppm and 20 ppm) and subjected to the same extraction protocol.

c) Volatile profiles of fermented milks

The aroma compounds in the fermented low-fat and soybean milk was detected by using the solid-phase microextraction and gas-chromatography-mass spectrometry (SPME-GC-MS) as reported by Patrignani et al. (2008). Samples, 5 g, were placed in 10 ml sterilized vials, sealed using PTFE/silicon septa and heated for 10 min at 45°C, after which the volatile compounds were allowed to adsorb to a fused silica fiber covered with a 75 µm carboxen polydimethylsiloxane (CAR/PDMS StableFlex) (Supelco, Steiheim, Germany). The adsorbed molecules were desorbed in the gas chromatograph for 10 min. The peaks were detected using an Agilent Hewlett-Packard 6890 GC gas chromatograph equipped with a 5970 MSD MS detector (Hewlett-Packard, Geneva, Switzerland) and a Varian Chrompack CP Wax 52 CB capillary column (50 m × 320 µm × 1.2 µm) (Chrompack, Middelburg, The Netherlands) as the stationary phase. The conditions used were as follows: injection temperature, 250◦C; detector temperature, 250°C; carrier gas (He); and flow rate, 1 ml/min. The oven-temperature program used was as follows: 50°C for 1 min; increasing from 50°C to 100°C at 2°C/min; increasing from 100°C to 200°C at 6.5◦C/min, and then holding at 200°C for 5 min. Volatile-peak identification was conducted via computerized matching of the mass spectral data with those for the compounds contained in the Agilent Hewlett-Packard NIST 98 and Wiley vers. Six mass spectral databases. The SPME-GC-MS results for each sample were expressed as the mean values of six independent analyses.

d) Rheological analysis of fermented milks

Textural properties of the fermented milks were evaluated at room temperature (21 ± 1°C) using an Instron Universal Testing Machine (Model4452, Instron Ltd, Wycombe, UK) equipped with a 500 N loadcell. Two different compression tests at a crosshead speed of 0.42 mm s^{-1}were carried out to obtain an in-depth textural characterization. Each sample (15 mm × 10 mm × 10 mm) was compressed by 30% of its initial height using a plunger with a plane, circular surface (58 mm

diameter). Texture profile analysis (TPA) was carried out by a double-compression method with a 5 s delay between the first and the second bite. Hardness, cohesiveness and springiness were determined according to Bourne (1978). Data reported are the average of ten replicates. A compression–relaxation test was carried out by compressing the sample up to a 20% maximum extent and then letting the cheese relax under compression for 120 s, after which the load was removed. The relative relaxation load was calculated as the ratio between the relaxation load and the maximum compression force. Data reported are the average of ten replicates. The elastic modulus (Y) was calculated as the slope of the stress–strain curve obtained from the first compression step of previously reported tests according to Rinaldi et al. (2010).

4.5.3. RESULTS AND DISCUSSIONS

a) EPSs production by strains in TPY with different carbon sources

In Table 13 the results about the quantification of viable cells after 24 hours of strains incubation in TPY added with 1.5 or 2% of glucose or lactose are showed.

	Glucose 1.5%	Dev.St.	Glucose 2%	Dev.St.	Lactose 1.5%	Dev.St.	Lactose 2%	Dev.St.
MRM_3.1	8.94	0.27	8.67	0.05	6.91	0.48	8.14	1.07
MRM_4.2	8.82	0.30	7.92	0.10	7.50	0.14	8.77	0.02
MRM_4.6	8.15	0.14	7.85	0.09	6.48	1.31	8.28	0.72
MRM_4.7	8.55	0.42	7.68	0.13	6.99	1.36	8.74	0.01
MRM_4.8	8.34	0.15	8.47	0.16		0.05	7.74	0.74
MRM_5.13	8.72	0.21	8.18	0.86	6.58	0.58	6.84	0.01
MRM_8.7	8.32	1.46	7.52	0.86	8.69	0.52	9.78	0.01
Re06	9.88	1.46	7.52	0.15	9.01	0.52	9.78	0.05
DSM 23967	8.92	0.42	7.90	0.23	8.14	0.10	8.93	0.00

Table 13. Viable counts expressed as log10 CFU/ml of TPY added with tested glucose and lactose concentrations. Standard deviations are also reported.

b) EPSs production by strains after milk fermentation

Table 14 shows the log change in viable cells counts of the *Bifidobacterium* strains in the fermented milks after incubation. The growth of tested bifidobacteria in different milk depended on the strain and on the matrix, however soy-milk resulted the favourite medium.

All starting inocula were high, however no significant changes in viable cells counts were observed after soy milk fermentation. Only the concentration of MRM_3.1 increased by more than 0,65 $\log_{10}$ CFU/ml, whereas MRM_4.8 and *B. saguini* DSM 23967^T showed a poor growth.

Low fat milk resulted as a hard medium for *B. aesculapii* strains. All strains suffer this environment showing a decrease in viable cells counts of more than 1 $\log_{10}$ CFU/ml, and the most sensitive strains were MRM_4.6 and MRM_4.7. Although there may be certain *Bifidobacterium* strains that can grow in un-supplemented milk, the general consensus is that *Bifidobacterium* strains do not grow well in milk. This is associated with a lack of essential vitamins or essential amino acids in milk. As a result, different compounds, such as yeast extract, bovine casein and serum albumin digests, and bovine milk whey, have been evaluated as growth-promoting factors for bifidobacteria. Only MRM_8.7 showed a slight increasing value, but it is affected by high standard deviation.

	Soybean milk inoculum	Dev. St.	Fermented Soybean milk	Dev. St.	Low-fat UHT milk inoculum	Dev. St.	Fermented Low-fat UHT milk	Dev. St.
MRM_3.1	8.77	0.10	9.26	0.32	9.39	0.06	8.24	0.34
MRM_4.2	9.08	0.25	8.83	0.08	9.32	0.09	9.08	0.02
MRM_4.6	9.82	0.62	9.24	0.40	9.37	0.09	7.80	0.42
MRM_4.7	9.34	0.11	9.92	1.23	9.47	0.12	7.64	0.39
MRM_4.8	9.17	0.18	8.84	0.09	9.62	0.08	8.12	0.33
MRM_5.13	9.41	0.07	10.06	1.18	9.50	0.04	9.22	0.31
MRM_8.7	8.96	0.38	9.06	0.38	9.40	0.32	9.58	1.13
Re06	-	-	8.57	0.24	9.94	0.04	9.03	0.52
DSM 23967	9.40	0.00	8.71	0.70	9.92	0.05	8.80	0.49

Table 14. Viable counts of inoculums and after fermentation for both soybean and low-fat milk expressed as log10 CFU/ml. Standard deviations are also reported.

c) EPSs extraction and quantification

The influence of carbon source (glucose 1,5%, 2% and lactose 1,5%, 2%) on the growth and EPSs production of tested strains was studied. Using glucose, all strains produced EPSs with the only exception for *B. saguini* at 2%. However, when lactose was used as the carbon source, the production of EPS dramatically decreased. Only four *B. aesculapii* strains produced EPS in 1,5% of lactose, whereas no EPS were quantified in 2% of lactose (Table 10).

The type and concentrations of carbon source has a huge influence on EPS productivity and *B. aesculapii* strains generally preferred glucose with differences according to the concentration. Indeed, the high EPSs productions and yields were obtained from MRM_3.1, and MRM_4.8 but at different concentrations, 1.5% (231.6 μg/ml) and 2%, (218.99 μg/ml) respectively (Table 10).

	Glucose 1.5% (μg/ml)	Glucose 2% (μg/ml)	Lactose 2% (μg/ml)	Fermented Soybean milk (μg/ml)	Fermented Low-fat UHT milk (μg/ml)
MRM_3.1	231.6	123.92	101.18	-	9.27
MRM_4.2	83.46	92.51	-	131.35	-
MRM_4.6	196.48	127.67	103.47	174.50	9.61
MRM_4.7	162.88	135.89		33.41	47.76
MRM_4.8	114.01	218.99	143.65	34.50	-
MRM_5.13	44.52	41.82	-	5.05	-
MRM_8.7	106.75	102.10	18.08	-	-
Re06	7.53	15.45	-	-	-
DSM 23967	12.88	-	-	-	-

Table 15. EPSs quantifications from the TPY added with glucose (1.5 and 2%) and lactose (2%), and the fermented soybean and low-fa milks.

The analysis of in situ EPSs production during low fat and soybean milk fermentation revealed that the matrix affected EPSs production. For instance, the highest numbers of EPS producers' strains was observed in soybean milk (Table 10). In fermented soybean milks, high EPSs production was recognized only for strains MRM_4.2 and MRM_4.6, 131.35 and 174.5 μg/ml, respectively.

Tested strains showed very low or no EPSs production after fermentation of low-fat milks. Strain MRM_4.7 was the highest producer with a yield of 47.76 μg/ml.

d) Volatile profiles of fermented milks

Volatile organic compounds (VOC) of fermented milk are quite complex; only a few compounds, mainly carbonyl compounds, such as acetaldehyde, acetone, 2-butanone, diacetyl, ethyl acetate, and ethanol, have a major effect on flavour development. In the present study, SPME-GC-MS was used to identify the VOC present in the milk fermentations and to detect their diverse distribution in different fermented products. The results of essential VOC profiles are summarized in Tables 11 and 12. Principal components analysis was performed to give an overall picture of the VOC distribution among the different fermented milk samples (Figure 9 and Figure 10).

	MRM_3.1	MRM_4.2	MRM_4.6	MRM_4.7	MRM_4.8	MRM_5.13	MRM_8.7	Re06	DSM 23967
Acetone	3.05E+06	3.05E+06	3.05E+06	8.50E+07	1.01E+08	1.32E+08	8.26E+07	1.01E+08	1.49E+08
Alcohol ethylic	5.31E+07	4.86E+07	5.06E+07	5.08E+07	1.12E+08	1.01E+07	4.40E+07	7.72E+07	3.97E+06
2,4 Dymethyl heptene	-	-	-	4.59E+07	6.30E+06	1.09E+07	7.32E+06	8.97E+06	2.89E+07
Ethyl acetate	-	-	-	1.89E+07	2.06E+07	2.31E+07	1.53E+07	1.80E+07	2.43E+07
2-Butanone	2.81E+06	2.26E+06	2.16E+06	2.80E+06	3.25E+06	5.56E+06	2.92E+06	3.09E+06	6.19E+06
2 -Ethyl furan	2.82E+06	2.95E+06	3.15E+06	3.15E+06	7.35E+06	5.43E+06	4.49E+06	7.41E+06	5.48E+06
2-Methyl heptanol	2.44E+07	2.36E+07	2.26E+07	2.04E+07	2.82E+07	4.12E+06	3.11E+07	4.70E+07	6.07E+06
Pentanone	1.65E+07	1.68E+07	1.98E+07	2.28E+07	9.82E+06	6.57E+06	7.55E+06	7.61E+06	1.20E+07
4-Ethyl 3 hexanolo	5.84E+06	1.06E+05	6.04E+06	6.44E+06	1.37E+07	1.11E+07	1.45E+07	8.91E+06	-
Heptanal	9.56E+06	-	9.76E+06	9.76E+06	3.49E+07	9.41E+06	2.97E+07	1.81E+07	-
2-Pentyl furan	5.58E+06	-	5.68E+06	5.68E+06	9.97E+06	9.46E+06	9.37E+06	8.55E+06	7.56E+06
2,3-Butanedione	1.05E+05	1.09E+05	1.09E+05	1.05E+07	6.37E+06	3.85E+06	1.46E+07	8.84E+06	4.30E+06
3-Hydroy 2 butanone	1.62E+07	1.52E+07	1.72E+07	1.52E+07	1.34E+07	4.76E+06	2.49E+07	1.47E+07	7.44E+06
Hexanal	2.01E+07	2.21E+07	2.51E+07	2.21E+07	3.07E+07	1.78E+07	3.06E+07	1.22E+07	3.74E+07
Standard	2.45E+07	2.45E+07	3.71E+07	-	-	-	-	-	-
3,7-Dymethyl decane	9.53E+06	-	-	9.83E+06	1.45E+06	3.57E+06	3.95E+06	6.09E+05	1.75E+06
3,6-Dymethyl decane	-	-	8.03E+06	8.33E+06	-	-	-	-	-
6,6-imethyl undecano	-	-	-	7.92E+06	3.74E+07	3.12E+07	4.29E+07	2.57E+07	4.09E+07
Octanal	1.23E+05	8.40E+03	-	3.43E+06	3.58E+06	5.07E+06	1.78E+06	1.61E+06	6.02E+06
Nonanal	5.59E+05	4.64E+05	5.54E+05	-	-	-	-	-	-
2-Hexyl decanolo	1.44E+07	1.33E+07	1.86E+07	1.56E+07	2.56E+07	1.25E+07	2.14E+07	1.68E+07	1.56E+07
Ethyl decanol	3.80E+06	4.00E+06	4.00E+06	4.60E+06	9.18E+06	-	-	-	.
Decanale	9.89E+06	1.05E+07	-	1.12E+07	1.20E+05	-	6.03E+06	-	-
Acetic acid	3.71E+08	3.81E+08	3.91E+08	4.11E+08	9.49E+06	1.81E+07	4.78E+08	1.58E+08	-
3-Heptaecanolo	7.11E+06	7.82E+06	7.05E+06	7.45E+06	1.21E+07	1.03E+07	9.25E+06	5.23E+06	1.64E+07
Benzaldehyde	4.05E+05	5.37E+04	8.42E+05	1.68E+07	2.04E+07	8.75E+06	2.56E+06	8.29E+06	1.54E+07
2-Tiophenmetanthiolo	8.29E+06	1.19E+06	2.78E+06	-	-	-	-	-	-
3-Decen 5 one	2.13E+05	1.52E+07	1.02E+07	1.52E+07	2.98E+06	7.40E+05	9.31E+05	2.04E+05	3.24E+06
1-Butanol 3 methyl formate	-	3.94E+06	3.72E+06	3.94E+06	1.77E+07	5.12E+06	1.30E+07	1.32E+07	1.01E+07
Tiophene 2 acetic acid dodecinil estere	1.61E+08	-	-	1.61E+08	1.26E+08	1.03E+08	1.12E+08	7.13E+07	1.56E+08
2,4 heptadienale 2,4 dimethyl	-	-	-	-	7.61E+06	5.95E+06	-	-	1.07E+07
1-Octen 3 olo	-	-	-	-	-	-	6.81E+05	-	6.20E+06
2-Methyl heptanol	-	-	-	-	-	-	-	-	1.58E+07
2-Nonen 1 olo	-	-	-	-	-	-	-	-	3.03E+07
2-Tiofenethanol	-	-	-	-	-	-	-	-	6.63E+07

Table 16. Volatile compounds in fermented soybean milks expressed as area. - =data under the detection limit.

The PCA analysis revealed a different volatile compound profile of fermented soybean milk from Re 06 if compared with those of *B. aesculapii* strains and *B. saguini* DSM 23967^T, along both the components (22% of variance). This strain is characterized for the production of 2-butatione, 3-heptaecanole and esanale. Interesting, fermented soybean milks from *B. aesculapii* MRM_4.7, MRM_4.8 and MRM_.13 are different for the presence of molecules normally characterizing the yoghurt aroma, such as 2,3-butandione.

Considering the volatile profiles from fermented law-fat milks, strain MRM_5.13 is different from all the others with high production of 2,3 butatione and 2-pentanone which allow is discrimination along the component 1, which explain the total variance of 25.4% (Fig. 1A and B)

	MRM_3.1	MRM_4.2	MRM_4.6	MRM_4.7	MRM_4.8	MRM_5.13	MRM_8.7	Re06	DSM 23967
Acetone	8.22E+06	7.00E+06	6.96E+06	8.66E+06	7.03E+06	1.76E+07	1.03E+07	5.90E+06	1.09E+07
Alcohol ethylic	1.24E+06	6.58E+05	6.30E+05	5.50E+05	-	2.42E+06	-	-	-
2,4 Dymethyl heptene	1.88E+06	1.51E+06	1.35E+06	1.62E+06	9.88E+05	2.28E+06	-	8.64E+05	-
Ethyl acetate	7.51E+06	7.63E+06	5.92E+06	6.19E+06	5.22E+06	1.25E+07	6.78E+06	3.60E+06	8.87E+06
2-Butanone	5.13E+06	4.83E+06	5.37E+06	2.51E+06	4.76E+06	7.57E+06	5.57E+06	8.94E+05	1.16E+06
2 -Ethyl furan	3.22E+06	3.02E+06	3.91E+06	3.95E+06	3.71E+06	5.43E+06	4.74E+06	2.57E+06	5.54E+06
2-Methyl heptanol	7.12E+05	6.92E+05	3.35E+05	1.10E+06	1.80E+06	-	-	-	-
Pentanone	2.39E+06	2.12E+06	-	1.23E+06	-	1.21E+06	-	-	-
4-Ethyl 3 hexanolo	3.87E+06	3.24E+06	4.99E+06	5.96E+06	5.27E+06	-	2.55E+06	2.42E+06	3.21E+06
Heptanal	6.22E+06	6.13E+06	3.79E+06	7.32E+06	1.68E+06	1.00E+06	2.79E+06	2.91E+06	4.97E+06
2-Pentyl furan	4.15E+06	3.87E+06	9.58E+06	6.58E+06	8.76E+06	6.08E+06	6.21E+06	5.97E+06	7.59E+06
2,3-Butanedione	9.36E+06	8.22E+06	2.23E+06	1.25E+07	8.18E+06	1.21E+07	4.66E+06	-	6.73E+06
3-Hydroy 2 butanone	4.65E+06	-	-	-	-	2.33E+05	-	-	-
Hexanal	1.41E+06	-	-	-	-	-	-	-	-
Standard	3.19E+06	1.25E+06	6.06E+06	4.63E+06	5.45E+06	-	5.08E+06	3.39E+06	5.50E+06
3,7-Dymethyl decane	3.26E+08	1.09E+08	2.66E+08	3.22E+08	3.22E+08	2.60E+07	2.32E+08	9.17E+07	1.75E+08
3,6-Dymethyl decane	4.80E+06	1.19E+06	-	2.38E+06	-	2.56E+06	3.57E+06	3.38E+06	4.70E+06
6,6-imethyl undecano	1.88E+06	1.13E+06	1.25E+06	1.01E+06	-	-	5.20E+05	-	-
Octanal	1.17E+07	2.46E+06	-	4.93E+06	7.58E+06	3.67E+06	3.74E+06	-	5.86E+06
Nonanal	2.50E+07	-	-	-	-	-	-	-	1.25E+07
2-Hexyl decanolo	9.65E+06	1.15E+07	2.66E+06	2.03E+07	1.38E+07	1.14E+07	-	3.64E+06	1.22E+07
Ethyl decanol	1.63E+07	4.63E+06	-	9.26E+06	3.94E+06	-	5.79E+06	-	1.04E+07
Decanale	1.00E+07	9.95E+06	1.38E+07	6.07E+06	5.30E+06	2.27E+07	1.91E+07	2.40E+06	1.53E+07
Acetic acid	2.24E+07	2.30E+07	1.79E+07	2.82E+07	2.44E+07	1.13E+07	2.61E+07	1.31E+07	2.62E+07
3-Heptaecanolo	9.34E+06	2.41E+06	4.82E+06	-	-	-	-	-	-
Benzaldehyde	-	6.86E+05	5.27E+05	8.44E+05	1.51E+06	1.74E+06	8.52E+05	9.34E+05	7.12E+05
2-Tiophenmetanthiolo	-	2.08E+05	4.17E+05	-	7.23E+05	5.80E+05	5.69E+05	6.92E+05	-
3-Decen 5 one	-	4.46E+06	7.87E+06	1.05E+06	1.45E+06	3.88E+06	7.10E+05	7.19E+05	6.65E+05
1-Butanol 3 methyl formate	-	1.80E+06	3.59E+06	-	7.12E+06	-	-	-	3.96E+06
Tiophene 2 acetic acid dodecinil estere	-	2.70E+07	5.40E+07	-	1.17E+06	-	-	-	-
2,4 heptadienale 2,4 dimethyl	-	4.42E+06	1.12E+06	7.72E+06	-	-	4.65E+06	4.93E+06	-
1-Octen 3 olo	-	2.42E+07	2.52E+07	2.32E+07	2.36E+07	1.79E+07	1.49E+07	1.06E+07	1.96E+07
2-Methyl heptanol	-	9.80E+06	1.10E+07	8.59E+06	5.86E+06	-	6.65E+06	-	7.81E+06
2-Nonen 1 olo	-	2.31E+06	5.97E+05	4.03E+06	-	-	-	-	-
2-Tiofenethanol	-	3.25E+06	6.50E+06	-	3.63E+06	-	1.28E+07	-	1.46E+07

Table 17. Volatile compounds in fermented low-fat milks expressed as area. - =data under the detection limit.

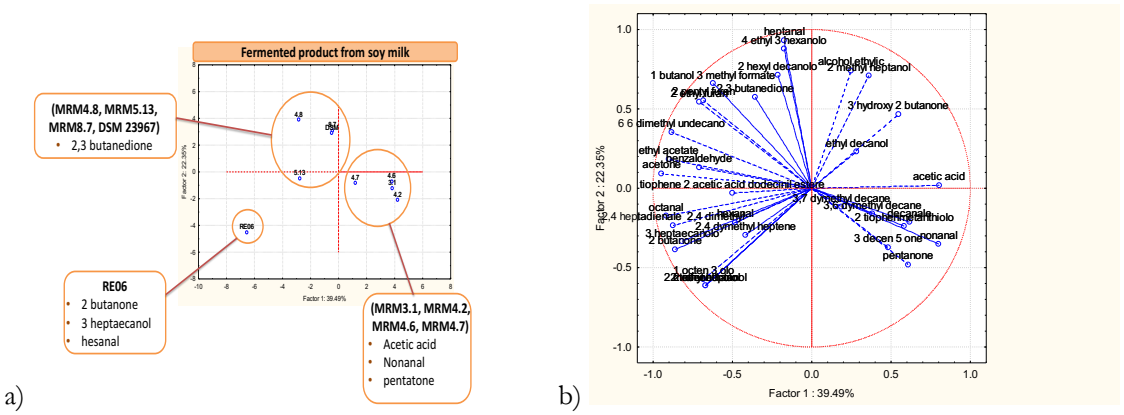

Figure 9. a) A projection of the cases (soybean fermented milks) on the factorial plane obtained by PCA on data about volatile organic compounds. b) Projection of variables (molecules) on the factorial plane.

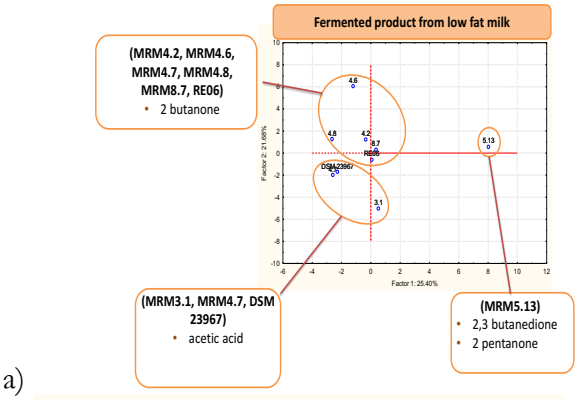

Figure 10. a) A projection of the cases (low-fat fermented milks) on the factorial plane obtained by PCA on data about volatile organic compounds. b) projection of variables (molecules) on the factorial plane.

e) Rheological analysis of fermented milks

The texture of any type of fermented milk product is important with regard to the quality of the products. It is related to sensory perception of food product. The most common sensory attributes relating to fermented milk texture are thickness /viscosity, smoothness (opposite to lumpiness, graininess, grittiness), and sliminess (or ropiness). Determination of the fermented milk texture usually includes sensory, structure and rheology analyses. Rheological parameters, such as firmness,

cohesion, adhesion and viscous index, were analysed for each fermented milk and results are summarized in Table 18 and Table 19.

Rheological profiles are very different, even scares results were retrieved from the negative, but also from the positive controls. As suggested from the EPSs quantification, strains were not able to grow well and produce extracellular compounds during fermentation of low fat milk, consequently rheological properties showed very low values. On the other hand, better results were obtained from the analysis of fermented soybean milks, which showed a better texture, probably due to the production of EPSs from the strains. In particular, MRM_4.7 showed the highest firmness value and viscous index (1071.2 g and 1071.2 g*s), but the less cohesion value, 23.96 g*s; while MRM_4.6 was able to produce the most cohesive and adhesive product (1399.55 g*s and 36.93 g) and it also showed good results for both the cohesion and the viscous indexes.

		Firmness g	Cohesion g*s	Adhesion g	Viscous Index g*s
MRM_3.1	Mean	27.71	550.43	8.25	4.50
	Dev. St.	14.14	277.40	1.09	4.61
MRM_4.2	Mean	29.61	600.97	11.97	15.17
	Dev. St.	15.15	279.91	4.18	18.16
MRM_4.6	Mean	63.27	1399.55	36.93	28.53
	Dev. St.	5.26	164.69	8.07	23.15
MRM_4.7	Mean	1071.42	23.96	10.04	1071.42
	Dev. St.	5.66	2.41	6.59	5.66
MRM_4.8	Mean	25.21	512.51	9.77	0.82
	Dev. St.	3.63	33.88	0.68	2.78
MRM_5.13	Mean	28.23	515.42	9.77	3.07
	Dev. St.	4.11	83.44	0.13	0.67
MRM_8.7	Mean	40.15	768.22	19.48	9.49
	Dev. St.	17.67	538.56	16.02	11.08
RE06	Mean	14.14	329.14	6.72	1.14
	Dev. St.	0.23	3.24	0.99	0.25
DSM23967	media	20.03	435.05	8.09	1.62
	dev. St.	0.98	40.48	0.94	0.36

Table 18. Rheological analysis results (firmness, cohesion, adhesion and viscous index) of fermented soybean milk.

		Firmness g	Cohesion g*s	Adhesion g	Viscous Index g*s
MRM_3.1	Mean	1.26	23.15	0.40	0.72
	Dev. St.	0.09	2.15	0.03	0.03
MRM_4.2	Mean	1.17	22.52	0.38	0.73
	Dev. St.	0.05	1.33	0.04	0.14
MRM_4.6	Mean	0.95	17.63	0.27	0.64
	Dev. St.	0.08	0.76	0.01	0.22
MRM_4.7	Mean	1.26	19.61	0.25	0.55
	Dev. St.	0.10	4.33	0.09	0.48
MRM_4.8	Mean	1.08	20.67	0.31	1.10
	Dev. St.	0.08	2.82	0.07	0.30
MRM_5.13	Mean	0.98	16.30	0.27	0.49
	Dev. St.	0.08	2.39	0.04	0.05
MRM_8.7	Mean	0.56	7.63	0.09	0.23
	Dev. St.	0.03	2.24	0.01	0.38
RE06	Mean	0.80	12.33	0.14	0.27
	Dev. St.	0.04	0.84	0.00	0.02
DSM23967	media	0.93	15.44	0.26	0.36
	dev. St.	0.03	0.69	0.02	0.08

Table 19. Rheological analysis results (firmness, cohesion, adhesion and viscous index) of fermented low-fat milk.

4.5.4. CONCLUSION

Based on the analysis performed and all the results obtained, soybean milk is a better matrix compared to the low-fat milk. Indeed, all *B. aesculapii* strains grew very well in soybean milk, producing considerable amounts of EPS, and resulted in high viscosity values.

Results about EPSs quantification suggested high yield in soybean samples for strains MRM_4.3, MRM_4.6, MRM_4.8 and MRM_5.13. Volatile organic compounds analysis revealed specific profiles and the texture showed a major viscosity.

Concluding, this work highlights the potential of *B. aesculapii* strains in enhancing aroma and texture of fermented soybean milk due to a high EPSs production.

4.6. FOLATE PRODUCTION

Folate, also called vitamin B9, is a water-soluble B vitamin that is wildly distributed in the biological world and it is naturally present in some foods, added to others, and available as a dietary supplement. Formerly known as folacin, folate is the generic term for both naturally occurring food folate and folic acid, the fully oxidized monoglutamate form of the vitamin that is used in dietary supplements and fortified foods (NIH, 2012).

Folate is a key molecule in (i) providing cells with the one-carbon units for metabolism of substances for replication and growth and (ii) it acts as a coenzyme in many important reactions, such as the interconversion of amino acids and the purine and pyrimidine nucleotides biosynthesis (McPartlin et al., 1999). (iii) Folate is also involved in the methylation of essential compounds (proteins, DNA and phospholipids) (Jacob, 2000).

Summarising, main folate functions are reduction of blood homocysteine levels, formation of red blood cells, folate is involved in the protein metabolism, cell growth and division and, in the first months of pregnancy, an adequate folate intake is necessary to prevent neural tube defects (NTD) and anencephaly.

4.6.1. THE IMPORTANCE OF FOLATE FOR HUMAN HEALTH

All this information empathizes the importance of folate as an important vitamin for our health; however, humans and other mammals are unable to synthetize it and they require an exogenous source of this vitamin (Hjortmo et al., 2005). A suggested folate intake of 600 μg/day DFEs (Dietary Folate Equivalents) are considered sufficient to maintain adequate folate status in pregnant women. Intake recommendations for folate and other nutrients are provided in the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (formerly National Academy of Sciences) (FNB, 1998).

The folate assimilation could derive from foods, such as breakfast cereals, fruits and fruit juices, nuts, beans, peas, dairy products, poultry and meat, eggs, seafood, grains green leaf vegetables and meat. The highest levels are found in spinach, liver, yeast extracts, asparagus, and Brussels sprouts (NIH, 2012). Nevertheless, this folate is instable and its presence is affected by appropriates conservation and cooking procedures. All these problems make difficult to achieve the optimal folate amount and an addition of synthetic folic acid is necessary. It is also to be considered that many authors reported a complex interaction between folic acid, vitamin B_{12} and iron and, a deficiency of one may be masked by the excess of the other, so the three substances must always be in balance (Allen et al., 1990; Reynolds, 2006; Vreugdenhil et al., 1990).

4.6.2. FOOD FORTIFICATION PROGRAM TO INCREASE FOLIC ACID INTAKES

The link between NTD and insufficient folic acid has increased the attention of governments and health organizations worldwide in giving recommendations concerning folic acid supplementation for woman intending to become pregnant. In the 1998, the U.S. Food and Drug Administration (FDA) developed a food fortification program to increase folic acid intakes and the blood folate levels in the population. The World Health Organization (WHO) and the Food and Agricultural Organization of the United Nations (FAO) defined fortification as "*the practice of*

deliberately increasing the content of an essential micronutrient, such as vitamins and minerals (including trace elements) in a food irrespective of whether the nutrients were originally in the food before processing or not, so as to improve the nutritional quality of the food supply and to provide a public health benefit with minimal risk to health…" (WHO), 2004).

There are four main different fortification types: (i) biofortification (breeding crops to increase their nutritional value, and can include both conventional selective breeding and modern genetic modification), (ii) synthetic biology (addition of probiotic bacteria), (iii) commercial and industrial fortification (flour, rice, oils, …) and (iv) home fortification (vitamin D drops). Many other countries have also established mandatory folic acid fortification programs, such as Canada, Costa Rica, Chile, and South Africa, but as 2013 no UE country (GP, 2013).

Indeed, fortification process is controversial; main issues are linked to the individual liberty, and potential undesirable effect on health (Smith, 2007). The second type of folate fortification, synthetic biology, could represent a social accepted alternative to other types of food fortification. Indeed, many microorganisms, including some intestinal bacteria, and higher plants are capable to bio-synthetize folate. Folate produced by intestinal microorganism could be absorbed by the host intestine (Krause et al., 1996) and could integrate the amount of this vitamin. It is also important because, in contrast to synthetized folic acid, bio-synthetized folate does not mask vitamin B_{12} deficiencies.

4.6.3. FOLATE PRODUCTION BY *BIFIDOBACTERIUM* SPP.

In the last decades, bifidobacteria have been more investigated for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, and colonic adenomas and cancer (Picard et al., 2005).

Due to their potential health benefits, members of *Bifidobacterium* genus belong to the group of probiotic bacteria, which have been defined in the work of ILSI Europe and the WHO (2001) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". In a few works, authors referred the folate production and gave information about accumulated cellular and secreted levels by different bifidobacteria strains (Lin and Young 2000; Crittenden et al. 2003; Pompei et al. 2007a,b; Strozzi and Mogna 2008).

Recently D'Aimmo et al. (2011) screened some bifidobacteria spp. for folate production and found high level of intra-cellular folate quantity; *B. catenulatum* ATCC 27539 resulted the best producer. The study by D'Aimmo et al. (2011) also highlighted a folate production and composition dependency from medium components and physiological state of microorganisms.

4.7. CASE OF STUDY 4. SCREENING OF FOLATE PRODUCTION FROM PRIMATES BIFIDOBACTERIA STRAINS AND SET-UP OF A HPLC QUANTIFICATION METHOD

Recommendations by health organizations have led to fortification programmes in many countries (LeBlanc et al., 2007), which have mandatory fortification of flour and uncooked cereal-grain products, such as USA, Canada, and Chile (D'Aimmo et al., 2012b). Other countries have

preferred not to fortify due to a potential link between high doses of synthetic folic acid and the development and progression of certain cancer forms (Hirsch et al., 2009; Mason et al., 2007) and due to the masking of vitamin B_{12} deficiency (Kim et al., 2004). Compared to the synthetic folic acid, natural folates, that are probably of lesser risk with respect to overdosing and cancerogenic risk (Kim et al., 2004), may be an alternative to fortification with synthetic folic acid.

Considering the importance of finding a more social accepted complementary source for folate intake, the biofortification with natural folates produced by selected microorganisms could be an interesting resource. Folate production from some strains belonging to the *Bifidobacterium* genus have been recently described (D'Aimmo et al., 2012b; Pompei et al., 2007). It seems reasonable to look for probiotic bifidobacterial strains able to increase not only the health of the consumers, but also the folate intake. Strains showed different production of folates and the comparison of folate production in bifidobacteria isolated from different animal host is a crucial step; indeed D'Aimmo *et al.* (2014) hypothesized a correlation between the capacity of the bifidobacteria to produce folate and the phylogenetic lineage of the host.

4.7.1. AIM OF THIS WORK

Aim of this study is the screening for the autotrophy for folate of fifteen strains isolated from common marmoset, ring tail and black lemur.

A high-performance liquid chromatography, HPLC, method has been utilized to quantify the production of different forms of folate, such as tetra-hydrofolate (THF) and methyl-5,6,7,8-tetrahydrofolate (5-CH_3-THF).

4.7.2. MATERIALS AND METHODS

Sixteen bifidobacterial strains were selected from those firstly isolated during this project (Table 20) ; *Bifidobacterium adolescentis,* strain ORG_4, was used as positive control.

Strain	Species	Isolation Source
MRM_5.9	*B. myosotis*	*Callithrix jacchus*
MRM_5.10	*B. myosotis*	*Callithrix jacchus*
MRM_5.18	*B. tissieri*	*Callithrix jacchus*
MRM_8.19	*B.* spp	*Callithrix jacchus*
MRM_9.3	*B.* spp	*Callithrix jacchus*
MRM_8.14	*B. hapali*	*Callithrix jacchus*
MRM_9.14	*B. hapali*	*Callithrix jacchus*
LMC_A13	*B. lemurum*	*Lemur catta*
LMM_E1	*B. lemurum*	*Eulemur macaco*
LMM_E2	*B. lemurum*	*Eulemur macaco*
LMM_E5	*B. lemurum*	*Eulemur macaco*
LMM_E3	*B. eulemuris*	*Eulemur macaco*
LMM_E13	*B. eulemuris*	*Eulemur macaco*
LMM_I1	*B. lemurum*	*Eulemur macaco*
LMM_I9	*B. lemurum*	*Eulemur macaco*
ORG_4	*B. adolescientis*	*Pongo pygmaeus*

Table 20. List of strains tested for autotrophic for folate. - = species not already described

The experiment includes a) the cultivation of strains in folate-free medium (FFM) for 4-5 subculturing, b) the extraction of the intra-cellular folate from autotrophic strains c) the set-up of the HPLC method, and d) the quantification of the H_4 folate and 5-CH_3-THF.

a) Screening the folate production capability

Strains were screened for the folate production according to D'Aimmo *et al.* (2012),. Briefly, the folate production was investigated sub-culturing the strains in FFM, which contain all the vitamins required for cells growth except folate.

Cells, obtained from freeze-dried cultures, were grown in TPY medium and incubated anaerobically at 37°C for 24 hours. The purity of the strains was checked before each analysis. After two sub-cultivations, cells were collected by centrifugation at 6000g for 20 min and washed twice with 0.9% NaCl, to eliminate traces of folate from the TPY medium.

Strains were subcultured in fresh FFM. After 10-12 hours (corresponding to the exponential phase of growth), the OD was measured and cultures showing OD values between 0.75 and 1.0 were sub-cultured in FFM anaerobically at 37°C. This protocol was carried out for 5 days. Strains showing growth after subculturing for 4-5 days were considered autotrophic for folate. After 4-5 days the bacterial cells were collected by centrifugation (6000 g, 15 min) and washed twice with cold 0.9% NaCl. The pellet was stored in the freezer (−80°C) and, when deeply frozen, freeze-dried for 2 days.

To verify the auxotrophy for folate for strains that were not growth, 5 mg/ml of folic acid were added to the FFM and their growth monitored for 5 days.

b) Extraction of folate from bifidobacteria

All the following procedures were conducted protecting samples from light and oxygen.

Cell extracts were prepared as described by D'Aimmo *et al.* (2012). Because low freeze-dried cell amount was obtained, the volume of each reagent was adjusted on the measured weight.

Briefly, 0.010 g of freeze-dried cells were suspended into 10 ml of a freshly prepared 0.1 mol l^{-1} phosphate buffer (pH 6.1) containing 2% ascorbic acid and 0.1% 2,3-dimercapto-1-propanol. Cells were boiled for 12 min in a water bath and cooled on ice; before recovering the supernatant by centrifugation (27000 g, 15 min, 4°C) and storing at −80°C, until the deconjugation of folate polyglutamates to monoglutamates. The deconjiugation step required dialysed rat serum deconjugase enzymes, which was prepared an stored at -80°C until the use. For the dialysis, the rat serum was provided by Scanbur, Uppsala (Sweden), and dialysed in 0.1 mol l^{-1} phosphate buffer containing 0.1% 2,3-dimercapto-1-propanol, at 4°C during stirring in dialysis tube (cut off 12000–14000 Da) for 3 h. The buffer was changed three times.

Aliquot of 50 µl of the dialysed rat serum was added to 1 ml of extracted sample in a glass tube to perform the deconjugation, which was carried out incubating the samples on a shaking water bath at 37°C for 3 h. To inactive the deconjugase enzymes extracts were boiled for 5 min and, after cooling on ice, the samples were recovered by centrifugation (27000g, 10 min, 4°C). The supernatants were analysed by HPLC.

c) Set-up of the HPLC method

Different folate forms could be measured in cell extracts, such as tetra-hydrofolate (THF) and methyl-5,6,7,8-tetrahydrofolate (5-CH_3-H_4 folate). Concentrations of intracellular folate was determined by HPLC following the method by Patring *et al.* (2005), which allows the detection of folate individual forms by UV (290 nm) and fluorescence detector (excitation 290 nm, emission 360 nm).

For the quantification of the different folate forms the (6S)-5,6,7,8-tetrahydrofolate, sodium salt ((6S)-H_4 Pte-Glu-Na_2) and the (6S)-5-methyl-5,6,7,8- tetra-hydrofolate, sodium salt (5-CH_3-H_4 Pte-Glu-Na_2) (Merk Eprova AG, Schaffhausen, Switzerland) were used as standards.

The purity of all standards was checked according to the procedure of Berg & Robijn (1995) using molar extinction coefficients and, after correcting quantification for purity, stocks solution of 200, 20 and 5 μg/ml in phosphate buffer were prepared avoiding oxygen by N_2 insufflation. Stock solutions were stored at -80°C protected from light. The working standard solutions were prepared the day of use elapsing the time before the standard is diluted in the final buffer.

d) Quantification of folate by HPLC

The quantification was performed according to D'Aimmo *et al.* (2012) and by using HPLC system, which consisted of a gradient quaternary pump (Jasco PU-2089 plus; Jasco, Mölndal, Sweden), a cooled autosampler (8°C) (Jasco AS-2057 plus), a UV detector (Chrompack) and a fluorescence detector (Jasco FP-920). The different forms of folate were detected by UV (290 nm) and fluorescence detector (excitation 290 nm, emission 360 nm). Both the HPLC systems and the processing of the data were controlled with the software Chromepass (Jasco, Mölndal, Sweden). The analytical column was Aquasil C18 150mm × 4.6 mm, 3 μm (Thermo Electron Corp., Västra Frölunda, Sweden) and the mobile phase consisted of 30 mmol l^{-1} phosphate buffer (pH 2.3) and acetonitrile. The acetonitrile gradient started at 6% for 5 min, thereafter increasing linearly to 25% in 20 min followed by an increase to 45% in 5 min, which was kept for another 5 min, finally back to the 6% in 1 min. The injection volume was 20 μl, and flow rate was 0.4 m min^{-1}.

4.7.3. Results and Discussions

The proposed demarcation of folate producing bifidobacteria based on their origin, indeed human and non-human origin, by D'Aimmo *et al.* (2014): In this paper bifidobacteria isolated from human sources showed autotrophy for folate differently from bifidobacteria isolated from non huma sources that showed auxotrophy for folate. In the present study this trend has not been confirmed. Nevertheless, more information should be collected and bifidobacteria from different sources should be investigated in order to clarify the correlation between bifidobacterial origin and folate production.

a) Screening the folate production capability

After revitalization from freeze-dried cultures, cells were twice sub-cultured in TPY broth, then bifidobacteria were tested for autotrophy in FFM (Table 21).

Strains from lemurs were not able to survive and no growth was observed after inoculation in the folate free media. On the other hand, some strains isolated from common marmoset, a more evoluted primate species, can grow in FFM, some of them for 2 and other for 4 days, when daily sub-cultivated in fresh FFM, except for MRM_9.3. However, the OD_{600} values, measured after 8 hours of incubation in FFM, revealed a difficult growth probably due to a low folate production, especially for MRM_5.9, MRM_5.10, MRM_5.18 and MRM_9.14.

MRM_8.14 and MRM_8.19 showed the same trend of the positive control ORG_4. For this strains the curve of growth spectrophotometrically monitored to identify the exponential phase is showed in Figure 11. These strains survived for 4 days of sub cultivation in FFM reaching the exponential phase, high OD values (more then 1.0), after 18 hour of incubation.

Strain	FFM		FFM+folate
	Day	OD	
MRM_5.9	2 days	0.4±0.02	5 days
MRM_5.10	2 days	0.37±0.01	5 days
MRM_5.18	3 days	0.41±0.12	5 days
MRM_8.19	4 days	1.06±0.06	5 days
MRM_9.3	no	-	5 days
MRM_8.14	4 days	1.06±0.06	5 days
MRM_9.14	2 days	0.37±0.03	5 days
LMC_A13	no	-	5 days
LMM_E1	no	-	5 days
LMM_E2	no	-	5 days
LMM_E5	no	-	5 days
LMM_E3	no	-	5 days
LMM_E13	no	-	5 days
LMM_I1	no	-	5 days
LMM_I9	no	-	5 days
ORG 4	4 days	1.38±0.20	5 days

Table 21. Results from strains growth in FFM and OD for autotrophic strains after 8 hours of incubation.

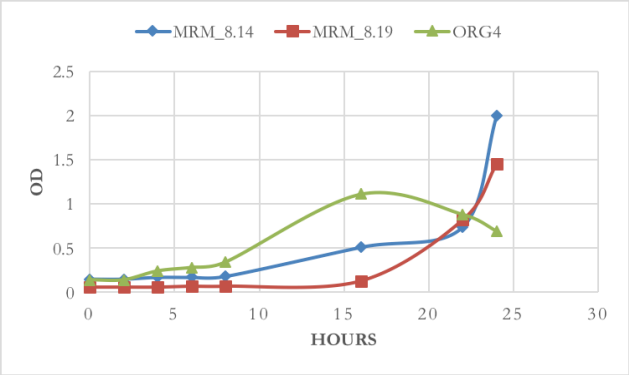

Figure 11. Curve of growth for strains MRM_8.14, MRM_8.19 and ORG_4.

b) Quantification of folate by HPLC

The intracellular folate of strains grown after subculturing for 4 days in FFM, was quantified by using HPLC system. About 0.005 and 0.016 mg of freeze dried cells (Table 22) were obtained from 20 ml of each culture: the experiment was conducted in triplicate.

Even if collected in the exponential phase and at more then 1 of OD, the free dry cell amount was low, indeed, 0.025 were required from the extraction method.

After intra cellular folate extraction and HPLC calibration, the quantifications of the two folate forms were performed and the total folate amount was calculated. All the quantification results, expressed as µg/100g of each folate form produced by each replicate of each strain are reported in Table 23 and graphically showed in Figure 12; histograms in Figure 13 displayed the mean folate forms produced by each strain.

SAMPLE (replicate)	Weight (g)
8.14 (1)	0,006
8.14 (2)	0,012
8.14 (3)	0,016
8.19 (1)	0,0104
8.19 (2)	0,005
8.19 (3)	0,009
ORG4 (1)	0,0112
ORG4 (2)	0,0094
ORG4 (3)	0,0113

Table 22. Weight of the cell recovered by centrifugation from 20 ml of each culure.

Sample	THF (µg/100g)	5-CH3-THF (µg/100g)	Total Folate (µg/100g)
MRM_8.14 (1)	242.98	431.22	674.20
MRM_8.14 (2)	668.74	724.06	1392.81
MRM_8.14 (3)	158.84	164.96	323.80
Mean	356.85	440.08	796.94
Dev.St.	273.36	279.66	544.97
MRM_8.19 (1)	75.50	350.62	426.13
MRM_8.19 (2)	59.66	404.60	464.26
MRM_8.19 (3)	29.50	316.62	346.12
Mean	54.89	357.28	412.17
Dev.St.	23.37	44.37	60.29
ORG4 (1)	111.59	336.87	448.46
ORG4 (2)	74.23	512.32	586.56
ORG4 (3)	36.77	296.91	333.69
Mean	74.20	382.04	456.23
Dev.St.	37.41	114.59	126.61

Table 23. Quantification of the folate produced by each sample; mean and standard deviation were calculated on triplicates for each strains. Values are expressed as µg/100g.

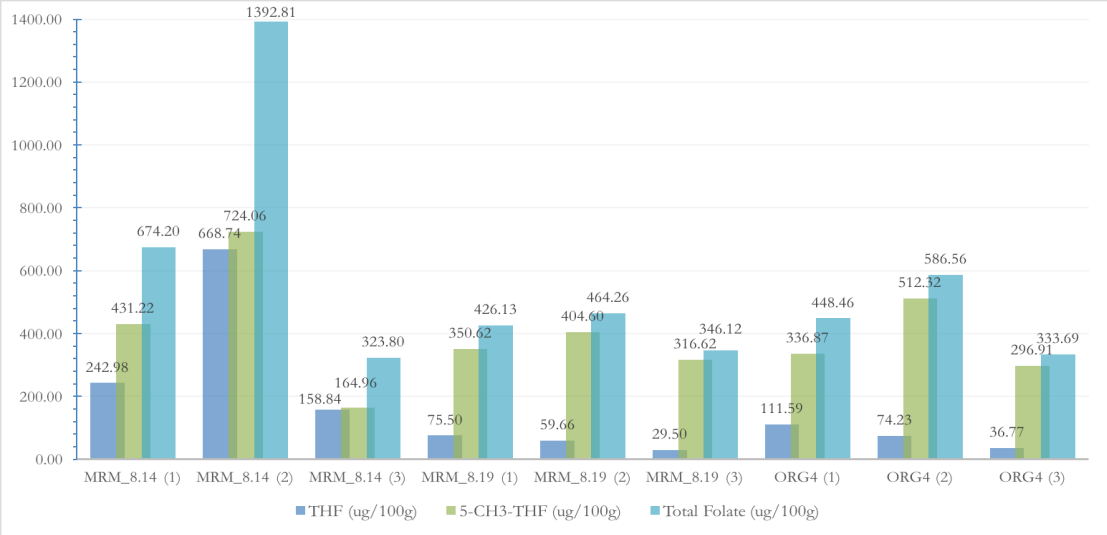

Figure 12. Histogram of the folate forms and total folate measured from each triplicate of each sample.

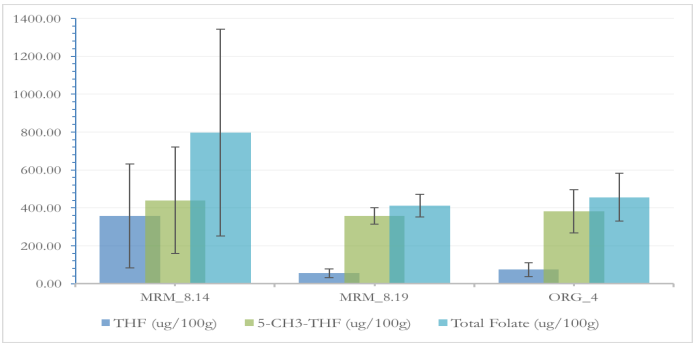

Figure 13. Histogram of the mean folate forms and mean total folate for each strain. Bars refers to the standard deviation of triplicates.

From our results, the 5-CH3-THF folate form seems the most produced by the bifidobacterial strains tested, ranging between a mean value of 357.28 and 440.08 μg/100g, compared to the THF folate form.

MRM_8.14 is the best folate producer strain, with a mean total folate value of 796.94 μg/100g, compared to the 456.23 μg/100g of the positive control, even if differences in the triplicates have been observed. Indeed, high standard deviation measured for each form of folate produced by MRM_8.14 did not support a good repeatability of the analysis suggesting a strong dependence of the folate production by the environmental conditions for this strain. High total folate and THF values of strain MRM_8.14 are affected by the quantification of the THF in the second replicate, 668.74 μg/100g, and it should be to reconsidered.

MRM_8.19 showed mean values similar to the positive control and also the lowest standard deviation. Basing on these quantifications, this strains could be selected for further investigation in order to confirm its autotrophy and its high folate production.

4.7.4. CONCLUSIONS

As reported by D'Aimmo *et al.* (2014) not all bifidobacteria possess the required machinery to synthetize folate *de novo* and the lineage of the host could be correlated with the capacity of the gut bifidobacteria to produce folate, rather than the type of diet.

However, selected bifidobacteria may potentially be used to either raise the level of folate in certain foods or as folatetrophic probiotics, that is, *in vivo* folate synthesis in the intestine, as shown in rats (Pompei et al., 2007) and humans (Strozzi and Mogna, 2008). Such application is dependent from knowledge about folate forms and quantities of folate produced by different bifidobacteria (D'Aimmo et al., 2012b).

In this work we screened for folate-autotrophy several bifidobacterial strains belonging to three non-human primate species, *Callithrix jacchus, Lemur catta* and *Eulemur macaco.* Results showed that not all strains are able to grow in the absence of folate and, in particular, only two strains belonging to two different species isolated from common marmoset can grow in the FFM for four subsequently subcultivations. All the other strains required external source of the vitamin as they showed autotrophy for folate.

Strains MRM_8.14, belonging to *B. hapali*, and MRM_8.19, belonging to a new bifidobacterial species not already described, are the only folate producer bifidobacteria among tested strains. The level of the intracellular folate quantified by HPLC was not high, ranging between 426.13 and 764.94 μg/100g; however, it could be compared to those produced by the positive control *B. adolescentis* strains ORG_4. As in the work by D'Aimmo *et al.* (2014), our results support the 5-CH3-THF as the most folate forms synthetized by bifidobacteria.

4.8. METHOD TO USE *Bifidobacterium* spp. AS FAECAL CONTAMINANT INDICATORS IN FOOD PRODUCTS

Setting up a method able to identify the fecal pollution source should be important both for assessing the degree of risk posed to public health and adequately addressing water quality problems (McLellan et al., 2003), but also to drive the development of strategies to mitigate the environmental loading of pathogens associated with waterborne disease transmission (Mohapatra et al., 2007).

4.8.1. FECAL INDICATOR BACTERIA (FIB)

In the last decades, different monitoring water and food products quality methods, based on measurement of Fecal Indicator Bacteria (FIB), i.e. Enterococci, faecal coliforms, *Escherichia coli* and *Clostridium perfringens*, were developed, such as membrane filtration (MF), chromogenic substrate, and quantitative PCR (qPCR) (Abdelzaher et al., 2010). FIB are easy to measure and have been found to correlate with human health out-comes (Prüss, 1998; Wade et al., 2003). FIB can originate from different pollution, and for monitoring purposes, it is important to possess knowledge of the possible dominant FIB sources. Several studies have focused on either phenotypic or genotypic characteristics of indicator bacteria such as streptococci or *Escherichia coli* (Carson et al., 2003; Dombek et al., 2000; Hagedorn et al., 1999; Harwood et al., 2000; Parveen et al., 1999; Wiggins et al., 1999). *Escherichia coli* is a microorganism normal inhabitant of the intestine of human and other warm-blooded animals, where is the predominant member of the facultative anaerobic group (Feng et al., 2002). *E. coli* belongs to the group of faecal coliforms and it is considered a specific indicator of faecal pollution in both water and other matrices. Indeed it generally does not survive outside the intestinal tract and its presence in environmental, food, or water samples should indicate a recent faecal contamination (Feng et al., 2002).

Numerous Microbial Source Tracking (MST) methods have been developed to distinguish between human or non-human fecal contamination source, and in some cases, among animal sources (Boehm et al., 2013). Many of them are library-dependent MST methods, which match genetic or phenotypic patterns of FIB isolates from a known source with isolates from a sample (Boehm et al., 2013). Indeed, to better represent *E. coli* population in a host group, the collection of isolates that broadly characterize single host groups is necessary (McLellan et al., 2003). The repetitive element anchored PCR (rep-PCR), which targets repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), or BOX elements, is a technique employed to compare bacterial genome diversity (McLellan et al., 2003). The identification of the pollution source is possible because, generally, a single animal harbours one predominant *E. coli* strain (McLellan et al., 2003).

Recently, animal associated genetic markers have been found to be geographically (Wiggins et al., 1999) and temporally specific (Jenkins et al., 2003), and have gained favour for independence from a library of known strains. Due to the extensive genetic structure within *E. coli* spp., Clermont et al. (2013) developed a quadruplex – PCR method to assigned *E. coli* isolates to one of seven phylogroups based on the use of four markers, genes and a DNA fragment.

4.8.2. BIFIDOBACTERIA AS FIB

As already discussed above, the habitats of bifidobacteria range from sewage to the intestines of humans, animals, and insects (Biavati et al., 1982; Scardovi and Trovatelli, 1969). In particular they were isolated from the feces of infants as well adults humans, many animals, such as ruminants, pigs, poultry, rodents, and rabbits, and social insects, fish and reptiles (Kopecný et al., 2010) representing a potential indicator of the faecal contamination of food products (Delcenserie et al., 2004).

Over other fecal contamination indicators, such as *E. coli*, many advantage are provided from the use of bifidobacteria in food products. Indeed, they are anaerobic stopping the growth in the presence of oxygen, but remain cultivable (Beerens et al., 2000), and it is therefore possible to estimate the initial amount of bifidobacteria present in the food product (Delcenserie et al., 2004). *Bifidobacterium* species are host specific, for example, *B. pseudolongum* subsp. *globosum*, *B. thermophilum*, and *B. boum* are present in ruminant feces (Klein et al., 1998); *B. longum* subsp. *suis* in swine; *B. cuniculi* and *B. magnum* in rabbit; *B. pullorum* in chicken; *B. adolescentis, B. dentium, B. bifidum, B. breve, B. catenulatum, B. longum* subsp. *longum* and *B. longum* subsp. *infantis* are present in the human intestine (Mangin et al., 1999).

This high specificity suggested the potential to determinate the origin of the contamination (animal or human) thought the determination of the bifidobacteria species. Several molecular methods have been developed for the identification of *Bifidobacterium* using different strategies. The 16S rDNA is a target commonly used for the bifidobacteria species identification by PCR to generate amplicon either by using species- specific primers (Matsuki et al., 2002; Ventura et al., 2001) or by using genus-specific primers followed by either sequencing (Miyake et al., 1998) or hybridization with species-specific probes (Lynch et al., 2002) or a PCR-restriction fragment length polymorphism (RFLP) (Mangin et al., 1999; Roy and Sirois, 2000; Ventura et al., 2001).

The last method represents an easy, slow and low cost approach already described by Delcenserie *et al.* (2004). The authors proposed two enzymes, *AluI* and *TaqI* to distinguish human- and animal borne strain; it has been developed on 64 strains belonging to 13 bifidobacteria species.

4.9. CASE OF STUDY 5. BIFIDOBACTERIA AS FAECAL CONTAMINATION INDICATORS: RECONSIDERING A PCR-RFLP METHOD TO DISTINGUISH HUMAN AND ANIMAL BIFIDOBACTERIA

Refers to **DRAFT 6.**

The aim of this work was the validation of the method by Delcenserie *et al.* (2004) in differentiating bifidobacteria from animal or human origin: an *in silico* restriction analysis on the available 16S rRNA gene sequences of all the currently described taxa of *Bifidobacterium* spp. and three type strains belonging to species currently under description was carried out.

4.9.1. AIM OF THIS WORK

The aim of this section was the validation of the method by Delcenserie *et al.* (2004) in differentiating bifidobacteria from animal or human origin. At that purpose, an *in silico* restriction analysis on the available 16S rRNA gene sequences of all the currently described taxa of *Bifidobacterium* spp. and three type strains belonging to species currently under description was carried out.

4.9.2. MATERIALS AND METHODS

Materials and Methods are accurately described in **DRAFT 6**.

Briefly, the 16S rRNA gene sequences of the 56 actually recognized bifidobacterial taxa were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) and also the 16S rRNA gene sequences of three novel bifidobacterial taxa recently isolated from non-human primates (*B. myosotis, B. hapali and B. tissieri)* were included. All the sequences were aligned using Clustal Omega and edited in the region flanked by primers described by Delcenserie *et al.* (2004).

All restriction analyses were performed *in silico* according to the method. As first step, the 16S rRNA partial gene sequences were digested with *AluI*. After patterns analysis the association with the different origin was performed with the support of a script written in Python (version 2.7.8) (https://www.python.org/) for this study. New patterns were labelled as New Profile (NP). Heterogeneous patterns were restricted with *TaqI*. After patterns analysis and origin attribution, additional unknown patterns were labelled NP. Presence of heterogeneous patterns was resolved searching for an alternative enzyme, *MaeIII*.

4.9.3. RESULTS AND DISCUSSIONS

Results and Discussions are fully reported in **DRAFT 6**.

Summarizing, as a result of the digestion with *AluI* of the all 16S rRNA edited gene sequences of bifidobacteria thirteen different patterns were obtained.

Therefore, restriction with *AluI* generated four heterogeneous groups (I, II, the new II-NP and the VII pattern, previously described as homogenous but now it is to be reconsidered) as including species of both human- and animal borne bifidobacteria.

The 16S rRNA sequences clustered in all heterogeneous profiles were restricted with *TaqI*. Two patterns, VIII and IX, previously identified and associated with the different origin were retrieved together with two new profiles now labelled as X-NP and XI-NP.

Analysing all the currently described species of bifidobacteria, *TaqI* was not able to correctly differentiate origin of some species in the groups II and II-NP. Therefore, the 16S rRNA sequences in those groups were further restricted by means of other several enzymes available in the CLC_Sequence Viewer database. Only the enzyme *MaeIII* resulted able to distinguish human from animal borne bifidobacteria and five homogenous groups were obtained.

Only for the species *B. scardovii*, it was not possible to discriminate the origin. Probably the source of this species need to be revised, as the strain was only isolated from female adult patients, viz. from 50-year-old female's blood sample in Sweden, from two elderly Swedish patients' urine sample, and from a 44- year-old female patient's hip (Hoyles et al., 2002).

4.9.4. CONCLUSIONS

Conclusions are more explain in **DRAFT 6.**

Bifidobacterium species are characterized by significant host specificity and seems that only by determining the *Bifidobacterium* species, one can also determine the origin of the strain (human or animal). Based on this hypothesis, Delcenserie *et al.* (2004) proposed the use of bifidobacteria as indicators of faecal pollution and developed a PCR-RFLP on the 16S rRNA gene to distinguish the origin (animal or human) of different bifidobacterial species. The method was tested in vivo on 64 strains belonging to 13 Bifidobacterium species. Currently, 49 species and six subspecies have been described. In this study, we verified the reliability of the method performing an *in silico* analysis on all the available 16S rRNA sequences of bifidobacterial validated type strains. We also included two novel species *Bifidobacterium* spp. MRM_8.19*, Bifidobacterium spp.* MRM_9.*3*, recently isolated from baby common marmosets. Results obtained from digestion with *AluI* showed nine homogenous patterns, including species of animal origin, and four heterogeneous patterns. Following Delcenserie *et al.* (2004), a second digestion with enzyme *TaqI* was performed on sequences in all heterogeneous groups. However, also *TaqI* originated two heterogeneous groups determining a third digestion with a new selected enzyme, *MaeIII*, which finally resulted able to correctly differentiate the origin of the species included in all heterogeneous patterns from *TaqI*.

Our study updated the method described by Delcenserie *et al.* (2004) confirming its ability to differentiate the origin (human, animal) of all currently validated bifidobacteria, even if three restriction enzymes are required.

DRAFT 6

Michelini, S., Modesto, M., Andlid, T. & Matarelli, P. Bifidobacteria as faecal contamination indicators: reconsidering a PCR-RFLP method to distinguish bifidobacteria derived from human and no-human primates.

Bifidobacteria as faecal contamination indicators: reconsidering a PCR-RFLP method to distinguish bifidobacteria derived from human and non-human gut

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Keywords: *Bifidobacterium* spp., PCR-RFLP, *AluI, TaqI, MaeIII,* fecal contamination indicator

ABSTRACT

In 2004, Delcenserie et al. proposed the use of bifidobacteria as a faecal contamination indicator, optimising protocols based on PCR-restriction fragment length polymorphism (PCR-RFLP) on the 16S rRNA gene, to identify different Bifidobacterium species sources. Two restriction enzymes, AluI, and Taq1, identified sixty-four strains as being of human and non-human origin from 13 species. Our aim was to validate this method by carrying out an in silico restriction analysis on 16S rRNA gene sequences of the 55 currently described taxa of the Bifidobacterium genus. Our results confirm the reliability of this method as a fast and simple strategy to determine both the presence and origin (human or non-human) of bifidobacteria. The protocol has been further optimized by the use of three restriction enzymes: AluI, Taq1 and MaeIII.

INTRODUCTION

Bifidobacteria are generally considered host-species-specific bacteria, and this has been validated by many studies (*2*). The reason for this close *Bifidobacterium* species - host relationship is unknown, but it is thought to be due to differences in the bifidobacterial cell-wall structures involved in intestinal epithelium adhesion, or to bifidobacterial ability to metabolize, in the intestine, specific substrates from the host diet. Interestingly, the species distribution of bifidobacteria in the faeces of human infants (*3*) and adults (*4*), the human vagina (*5*) and dental caries (*6*) also indicates adaptation differences by species of human origin in different habitats of the same host. Indeed, on investigating different hosts, not just humans, the bifidobacterial occurrence and species composition clearly suggest separate non-human bifidobacteria. The following species have, to date, been found only in human beings: *B.*

adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. dentium, B. gallicum, B. longum subsp. *longum*, *B. longum* subsp. *infantis, B. pseudocatenulatum,* and *B. scardovii*. On the other hand the following species have been associated exclusively with non-human hosts: *B. cuniculi* and *B. magnum* have been found only in rabbit faecal samples, *B. gallinarum* and *B. pullorum* only in the intestine of chicken, *B. longum* subsp. *suis* and *B. longum* subsp. *suillum* only in piglet faeces (*7*), *B. thermacidophilum* subsp. *Porcinum* and *B. thermophilum* in swine (*8*). *B. asteroides* is the only species found in *Apis mellifera* intestine, while *A. cerana* and *A. dorsata* harbor the species *B. indicum* (*8*). Finally, *B. actinocoloniforme, B. bohemicum,* and *B. bombi* are present in *Bombidae*. To date, *B. aesculapii* (*9*)*, B. biavati, B. callitrichos, B. reuteri* and *B. stellenboschense* (*10*)*, B. myosotis, B. tissieri* and *B. hapali (10)*, *B. moukalabense (13)*, *Bifidobacterium spp*. strain MRM_8.19 and *Bifidobacterium spp*. strain MRM_9.3 (*11*) have been found only in non-human primates. However, *B. breve* and *B. longum* subsp. *Infantis* have been found harbored in the faeces of suckling calves and human breastfed infants, not the only case where species typical of a human habitat have been found in non-humans. Indeed, the bifidobacterium "*Bifidobacterium angulatum like"* (*12, 13*) has been found in the Ape family, in chimpanzees (*14*), while *Bifidobacterium dentium* and *Bifidobacterium adolescentis* are in chimpanzee and orangutan (*14*) respectively. This has led to the suggestion that the determination of the *Bifidobacterium* species would make it possible to define the human or non-human origin of the strain (*1*). Indeed, several studies have proposed rapid identification methodologies for environmental *Bifidobacterium* strains to discriminate between human and non-human origins. Scardovi et al. (*15*) analysed the electrophoretic mobility of the fructose-6-phosphate phosphoketolase enzyme and concluded that its mobility varies according to the species (*16*). Gavini et al.*(18)* found that growth at 45°C in Trypticase-phytone-yeast extract (TPY) broth seems to provide good discrimination between strains derived from humans and non-humans: indeed, whereas the non-human strains were able to grow at 45°C or higher, most of the human strains could not *(17)*. Mara and Oragui *(19)* described a selective medium, Human Bifid-Sorbitol agar, which was able to isolate sorbitol-fermenting strains. As sorbitol is a food additive in products for human consumption, sorbitol- fermenting *Bifidobacteria* (SFB) are most likely of human origin (*18*). However SFB have also been detected in pig faeces (*17*). Finally, folate production in bifidobacteria shows folate autotrophy to be closely related to human bifidobacterial species, whereas the non-human species does not grow without folate addition to a synthetic folate free medium *(15)*. *Bifidobacterium* identification by molecular methods is typically performed using 16S rRNA sequence analysis. The amplicon, when generated using genus specific primers, can be either sequenced or subjected to restriction analysis. The most extensive PCR-RFLP study on *Bifidobacterium* species, based on 16 rDNA, was done by Ventura et al. (*19*). Sixteen species were investigated using the enzymes *Sau*3AI and *Bam*HI. However, some species were not investigated (*B. merycicum, B. ruminantium, B. minimum,* and *B. thermophilum*).

In a subsequent study, Delcenserie et al. (*1*), after designing specific PCR primers matching the 16S rDNA region, classified sixty-four strains belonging to thirteen *Bifidobacterium* species by means of the *Alu*I enzyme. This restriction led to seven different groups, but as two groups contained both human and non-human strains the *Taq*I enzyme was used to correctly differentiate the strain origin. Thus, the first description of the 16S rRNA PCR-RFLP method was as a molecular tool to speed up bifidobacterial discrimination, especially for a definition of human or non-human origin, such as in Microbial Source Tracking (MST) studies and probiotic selection.

In this study, the method proposed by Delcenserie et al. (*1*) was applied to all 55 *Bifidobacterium* type strains described to date, and to three strain types of new species currently under description. Our aim was to verify the method's reliability in differentiating bifidobacteria of human or non-human origin, performing *in silico* restriction analysis on the available 16S rRNA gene sequences.

MATERIALS AND METHODS

Bifidobacterium 16S rRNA partial gene sequences

The 16S rRNA gene sequences of the 55 recognized bifidobacterial taxa were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/), and are listed in Table 1. We also included the 16S rRNA gene sequences of three novel bifidobacterial taxa recently isolated from non-human primates. All the sequences were first aligned in CLC_Sequence Viewer version 7.5, for Mac OS (CLC, Inc., Aarhus, Denmark) using Clustal Omega, and then edited in the region flanked by primers 16S direct, 5' – AAT AGC TCC TGG AAA CGG GT – 3', and 16S reverse, 5' - CGT AAG GGG CAT GAT GAT CT – 3' (Delcenserie et al. 2004). Final sequences of about 1050 bp were obtained.

Table 1. List of species (all type strains unless specified otherwise), origin, international collection and GenBank accession number and fragment size (in bp) for each partial 16S rRNA gene sequence used in this study.

Species	Origin	Collection Nr.	GenBank Accession Nr.	16S rRNA fragment size (bp)
B. actinocoloniforme	Non-human	DSM 22766	FD858731	1054
B. adolescentis	Human	DSM 20089	AB437355	1056
B. aesculapii	Non-human	DSM 26737	KC807989	1055
B. angulatum	Non-human	ATCC 27535	D86182	1054
B. animalis subsp. *animalis*	Non-human	JCM 1190	D86185	1066
B. animalis subsp. *Lactis*	Non-human	DSM 10140	AB050136	1064
B. asteroids	Non-human	DSM 20089	EF187235	1052
B. biavatii	Non-human	DSM 23969	AB559506	1062
B. bifidum	Human	DSM 20456	AB437356	1054
B. bohemicum	Non-human	DSM 22767	FD858736	1053
B. bombi	Non-human	DSM 19703	HE582780	1051
B. boum	Non-human	JCM 1211	D86190	1054
B. breve	Human	ATCC 15700	AB006658	1056
B. callitrichos	Non-human	DSM 23973	AB559503	1051
B. catenulatum	Non-human	DSM 16992	AB437357	1054
B. choerinum	Non-human	ATCC 27686	D86186	1064
B. commune	Non-human	DSM 28792	LK054489	1051
B. coryneforme	Non-human	DSM 20216	AB437358	1052
B. crudilactis	Non-human	DSM 20435	NR_115342	1050
B. cuniculi	Non-human	DSM 20435	AB438223	1065
B. dentium	Human	ATCC 27534	D86183	1056
B. faecale	Non-human	JCM 19861	KF990498	1055
B. gallicum	Non-human	JCM 8224	D86189	1064
B. gallinarum	Non-human	JCM 6291	D86191	1050
B. hapali	Non-human	JCM 30799	KP7189460	1057
B. hapali	Non-human	JCM 30800 (Reference Strain)	KP7189462	1055
B. indicum	Non-human	JCM 1302	D86188	1052
B. kashiwanohense	Non-human	DSM 21854	NR_112779	1053
B. lemurum	Non-human	DSM 28807	KJ658281	1052
B. eulemuris	Non-human	JCM 30801	KP979748	1051
B. longum subsp. *infantis*	Human	ATCC 15697	D86184	1051
B. longum subsp. *longum*	Human	ATCC 55813	DB437359	1051
B. longum subsp. *suis*	Non-human	ATCC 27533	M58743	1051
B. magnum	Non-human	JCM 1218	D86193	1062
B. merycicum	Non-human	JCM 8219	D86192	1054
B. minimum	Non-human	DSM 20102	AB437350	1051
B. mongoliense	Non-human	DSM 21395	AB433856	1051

B. moukalabense	Non-human	JCM 18751	AB821293	1059
B. myosotis	Non-human	JCM 30796	KP718941	1051
B. myosotis	Non-human	JCM 30797 (Reference Strain)	KP718942	1051
B. pseudocatenulatum	Human	JCM 1200	D86187	1054
B. pseudolongum subsp. *globosum*	Non-human	DSM 20092	M58736	1065
B. pseudolongum subsp. *pseudolongum*	Non-human	JCM 1205	D86195	1064
B. psychraerophilum	Non-human	DSM 22366	AB437351	1050
B. pullorum	Non-human	JCM 1214	D86196	1051
B. reuteri	Non-human	DSM 23975	AB613259	1054
B. ruminantium	Non-human	JCM 8222	D86197	1056
B. saeculare	Non-human	DSM 6531	D89328	1051
B. saguini	Non-human	DSM 23967	AB559504	1052
B. scardovii	Human	DSM 13734	N180852	1052
Bifidobacterium spp.	Non-human	BUSCOB MRM_8.19	KP7189459	1054
Bifidobacterium spp.	Non-human	BUSCOB MRM_9.3	KP7189460	1053
B. stellenboschense	Non-human	DSM 23968	AB559505	1060
B. subtile	Non-human	DSM 20096	D89378	1052
B. thermacidophilum subsp. *porcinum*	Non-human	DSM 17755	AB437361	1054
B. thermacidophilum subsp. *suis*	Non-human	DSM 17775	NR 025672	1054
B. thermacidophilum subsp. *thermacidophilum*	Non-human	DSM 15837	AB437362	1054
B. thermophilum	Non-human	DSM 20210	AB437364	1054
B. tissieri	Non-human	JCM 30798	KP7189451	1053
B. tissieri	Non-human	JCM 30803 (Reference Strain)	KP7189457	1053
B. tsurumiense	Non-human	DSM 17777	AB241106	1056

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; BUSCOB, Bologna University Scardovi Collection of Bifidobacteria.

Restriction enzyme analysis

All restriction analyses were performed *in silico* using the tool Restriction Site Analysis available in CLC_Sequence Viewer version 7.5, for Mac OS (CLC, Inc., Aarhus, Denmark). In accordance with the method proposed by Delcenserie et al. (2004), the 16S rRNA partial gene sequences were digested with *AluI*. Each pattern was analysed and compared to the groups previously described and associated with the different origins by Delcenserie et al. (2004), using a script written in Python (version 2.7.8) (https://www.python.org/) (Supplementary File 1) for this study. If an unknown restriction profile was obtained, it was labelled as New Profile (NP) and the origin of the corresponding bifidobacterial species was recognized. When a heterogeneous pattern was obtained, the corresponding 16S rRNA gene sequences were restricted with *TaqI* or alternatively, if necessary, with *MaeIII* until the right origin was obtained.

RESULTS AND DISCUSSION

Restriction analysis of the 16S rRNA partial gene sequence with *AluI* or *TaqI* has been described as an easy way to distinguish between human and non-human borne bifidobacteria (*I*). The method was previously tested on sixty-four strains belonging to only 13 *Bifidobacterium* species, but nowadays, 49 species and 6 subspecies have been validated. To verify the reliability of this method, *in silico* restriction analysis was performed on the 16S rRNA partial gene sequences of 55 validated type strains. We also included three strains belonging to two putative novel *Bifidobacterium* species isolated from baby common marmosets (*11*) and to recently described novel species from an adult subject of black lemur (*20*).

All the aligned 1050 bp sequences belonging to the 55 bifidobacterial type strain were first digested with the enzyme *AluI* (Table 2), and thirteen different patterns were obtained. Seven of these patterns were also found by Delcensiere et al. (*1*), while the others were labelled as New Profile and added to the Python script, which was able to rapidly calculate each restriction profile and associate it with the respective group (Table 2).

Table 2. *AluI_1* restriction profiles obtained for each species with information about the pattern attribution and the origin.

Species	Frag1	Frag2	Frag3	Frag4	Frag5	Frag6	*AluI_1* pattern	Origin
B. animalis subsp.*lactis*	814	134	96	6			I	Human or Non-human
B. cuniculi	814	133	97	6			I	Human or Non-human
B. animalis subsp.*animalis*	815	132	97	6			I	Human or Non-human
B. stellenboschense	810	137	97	6			I	Human or Non-human
B. aesculapii	806	142	96	6			I	Human or Non-human
B. adolescentis	806	142	96	6			I	Human or Non-human
B. reuteri	804	144	96	6			I	Human or Non-human
B. myosotis^{T}	492	406	146	6			I-NP	Non-human (Common marmoset)
B. myosotis (reference strain)	492	406	146	6			I-NP	Non-human (Common marmoset)
B. asteroides	596	206	146	96	6		II	Human or Non-human
B. scardovi	598	206	145	95	6		II	Human or Non-human
Bifidobacterium spp. MRM 8.19	598	206	144	96	6		II	Human or Non-human
B. actinocoloniforme	598	206	144	102	0		II	Human or Non-human
B. tsurumiense	600	206	142	96	6		II	Human or Non-human
Bifidobacterium spp. MRM_9.3	597	206	144	97	6		II	Human or Non-human
B. tissieri^{T}	597	206	145	96	6		II	Human or Non-human
B. tissieri (reference strain)	597	206	145	96	6		II	Human or Non-human
B. dentium	600	206	142	96	6		II	Human or Non-human
B. bifidum	598	206	144	96	6		II	Human or Non-human
B. kashiwanohense	598	206	145	95	6		II	Human or Non-human
B. biavati	606	206	136	96	6		II	Human or Non-human
B. pseudolongum subsp.*globosum*	608	206	133	97	6		II	Human or Non-human
B. choerinum	608	206	134	96	6		II	Human or Non-human
B. pseudolongum subsp. *pseudolongum*	608	206	134	96	6		II	Human or Non-human
B. breve	601	206	142	95	6		II	Human or Non-human
B. longum subsp. *longum*	596	206	147	60	35	6	II-NP	Human or Non-human
B. gallinarum	595	206	148	60	35	6	II-NP	Human or Non-human
B. saeculare	596	206	147	60	35	6	II-NP	Human or Non-human
B. longum subsp. *suis*	596	206	147	60	35	6	II-NP	Human or Non-human
B. longum subsp. *infantis*	596	206	147	60	35	6	II-NP	Human or Non-human
B. pullorum	596	206	147	60	35	6	II-NP	Human or Non-human

B. magnum	607	206	136	60	35	6	II-NP	Human or Non-human
B. merycicum	408	286	206	144	6		III	Non-human
B. callitrichos	405	286	206	147	6		III	Non-human
B. angulatum	408	286	206	144	6		III	Non-human
B. hapalii^{T}	315	286	206	140	60	37	III-NP	Non-human
B. hapalii (reference strain)	313	286	206	142	60	37	III-NP	Non-human
B. bombi	310	286	206	147	60	35	III-NP	Non-human
B. eulemuris	310	286	206	147	60	35	III-NP	Non-human
B. ruminantium	902	142	6				IV	Non-human
B. bohemicum	310	246	207	145	102	40	IV-NP	Non-human
B. mongoliense	310	286	206	147	95	6	V	Non-human
B. psychraerophilum	310	285	206	148	95	6	V	Non-human
B. crudilactis	310	285	206	148	95	6	V	Non-human
B. subtile	310	286	206	146	96	6	V	Non-human
B. commune	310	286	206	147	95	6	V	Non-human
B. coryneforme	310	286	206	146	96	6	V	Non-human
B. minimum	310	286	206	147	95	6	V	Non-human
B. indicum	310	286	206	146	96	6	V	Non-human
B. lemurum	352	311	286	60	35	6	V-NP	Non-human
B. moukalabensis	699	206	139	6			VI	Human
B. pseudocatenulatum	694	206	144	6			VI	Human
B. catenulatum	694	206	144	6			VI	Human
B. gallicum	375	233	206	134	96		VI-NP	Human
B. thermophilum	805	144	60	35	6		VII	Non-human
B. boum	805	144	60	35	6		VII	Non-human
B. thermacidophilum subsp. *thermacidophilum*	805	144	60	35	6		VII	Non-human
B. saguini	804	145	60	35	6		VII	Non-human
B. faecale	806	143	60	35	6		VII	Non-human
B. thermacidophilum subsp. *suis*	805	144	60	35	6		VII	Non-human
B. thermacidophilum subsp. *porcinum*	805	144	60	35	6		VII	Non-human

The following patterns are shown: pattern I (800– 150–100 bp) included *B. animalis* subsp. *animalis, B. animalis* subsp. *lactis*, *B. cuniculi*, *B. stellenboschense*, *B. aesculapii*, *B. reuteri* and *B. adolescentis*; pattern II (600–200– 150–100 bp) included *B. asteroides, B. scardovii, B. acticoloniforme, B. tsurumiense, B. tissieri, B. kashiwanohense, B. biavatii, B. choerinum, B. pseudolongum* subsp. *globosum*, *B. pseudolongum* subsp. *pseudolongum*, *B. bifidum*, *B. breve*, and *B. dentium,* strains *Bifidobacterium* spp. MRM 8.19 and *Bifidobacterium* spp. MRM 9.3; pattern III (400–300–200–150 bp) included *B. merycicum, B. angulatum* and *B. callitrichos*; pattern IV (900–150 bp) included *B. ruminantium;* pattern V (310–290–200–150– 100 bp) included *B. minimum, B. indicum, B. coryneforme, B. commune, B. subtile, B. crudilactis, B. psychraerophilum, B. mongoliense*; pattern VI (700–200–150 bp) included *B. pseudocatenulatum, B. catenulatum* and *B. moukalabense*; and pattern VII (800–150–50–30) included *B. thermophilum, B. boum, B. thermoacidophilum* subsp. *thermoacidophilum, B. thermoacidophilum* subsp. *suis, B. thermoacidophilum* subsp. *porcinum, B. saguini, B. faecale.*

The following new patterns were shown: pattern I-NP (492-406-146-6), IV-NP (310-246-107-145-102-40), V-NP (352-311-286-60-35-6) and VI-NP (375-233-206-134-96) each included one species: *B. myosotis* (type and reference strains), *B. bohemicum*, *B. lemurum* and *B. gallicum,* respectively. Furthermore, group III-NP (315-286-206-140-60-37) included three non-human species: *B. bombi*, isolates from insects, *B. hapali* (type and reference strains), recently isolated from baby common marmosets and *B. eulemuris*, a novel species isolated from the black lemur (*20*). However, pattern II-NP (590/600-206-145-60-35-6 bp) included *B. longum* subp. *suis*, *B. magnum*, *B. pullorum*, *B.*

saeculare, B. longum subp. *infantis, B. longum* subsp. *longum* and *B. gallinarum,* then resulted heterogeneous.

Therefore, restriction with *AluI* generated four heterogeneous groups (I, II, the new II-NP and the VII pattern, previously described by Delcenserie et al. (*1*) as homogenous, differently from this study) including species of both human- and non-human borne bifidobacteria.

As the aim of the method was to distinguish bifidobacteria with respect to origin, the second enzyme *TaqI* was used for the restriction of the 16S rRNA sequences clustered in all the heterogeneous profiles. Resulting profiles were elaborated with the Python script: two patterns, VIII and IX, previously identified and associated with different origins by Delcenserie et al. (*1*) were retrieved, together with the two new profiles X-NP and XI-NP (Table 3). Based on the new fragment length profiles calculated, species of groups I, II and II-NP were reassigned to groups VIII and IX, X-NP and XI-NP.

Table 3. *TaqI* restriction profiles obtained for each species with information about the pattern attribution and the origin.

Species	Frag1	Frag2	Frag3	Frag4	*TaqI* pattern	Origin
B. animalis subsp. *animalis*	0	238	341	471	VIII	Non-human
B. animalis subsp. *lactis*	0	240	339	471	VIII	Non-human
B. boum	0	250	330	470	VIII	Non-human
B. choerinum	0	240	339	471	VIII	Non-human
B. cuniculi	0	239	340	471	VIII	Non-human
B. pseudolongum subsp. *globosum*	0	239	340	471	VIII	Non-human
B. pseudolongum subsp. *pseudolongum*	0	240	339	471	VIII	Non-human
B. saguini	0	251	328	471	VIII	Non-human
B. tissieri^{T}	0	251	328	471	VIII	Non-human
B. tissieri (reference strain)	0	251	328	471	VIII	Non-human
Bifidobacterium spp. MRM_8.19	0	250	329	471	VIII	Non-human
Bifidobacterium spp. MRM_9.3	0	250	328	472	VIII	Non-human
B. asteroides	134	193	252	471	IX	reconsidered as Human or Non-human
B. dentium	134	197	248	471	IX	reconsidered as Human or Non-human
B. bifidum	134	195	250	471	IX	reconsidered as Human or Non-human
B. breve	133	198	248	471	IX	reconsidered as Human or Non-human
B. adolescentis	134	197	248	471	IX	reconsidered as Human or Non-human
B. faecale	133	197	249	471	IX	reconsidered as Human or Non-human
B. kashiwanohense	133	195	251	471	IX	reconsidered as Human or Non-human
B. tsurumiense	134	197	248	471	IX	reconsidered as Human or Non-human
B. magnum	133	204	242	471	IX	reconsidered as Human or Non-human
B. thermophilum	133	197	250	470	IX	reconsidered as Human or Non-human

B. thermacidophilum subsp. *thermacidophilum*	133	197	250	470	IX	reconsidered as Human or Non-human
B. thermacidophilum subsp. *suis*	133	197	250	470	IX	reconsidered as Human or Non-human
B. thermacidophilum subsp. *porcinum*	133	197	250	470	IX	reconsidered as Human or Non-human
B. aesculapii	0	0	249	801	X-NP	Non-human (Monkey)
B. reuteri	0	0	250	800	X-NP	Non-human (Monkey)
B. longum subsp. *suis*	0	133	253	664	XI-NP	Non-human or Human
B. longum subsp. *infantis*	0	133	253	664	XI-NP	Non-human or Human
B. longum subsp. *longum*	0	133	253	664	XI-NP	Non-human or Human
B. actinocolinoforme	0	134	250	666	XI-NP	Non-human or Human
B. scardovii	0	133	251	666	XI-NP	Non-human or Human
B. stellenboschense	0	135	243	672	XI-NP	Non-human or Human
B. biavatii	0	134	242	674	XI-NP	Non-human or Human
B. gallinarum	0	133	254	663	XI-NP	Non-human or Human
B. pullorum	0	133	253	664	XI-NP	Non-human or Human
B. saeculare	0	133	253	664	XI-NP	Non-human or Human

Groups VIII and X-NP resulted homogeneous, including only species of non-human origin: group VIII (471-340-240) included *B. animalis* susbp. *animalis*, *B. animalis* subsp. *lactis*, *B. choerinum*, *B. cuniculi*, *B. pseudolongum* subsp. *pseudolongum*, and *B. pseudolongum* subsp. *pseudolongum* while group X-NP (800-250 bp) included two non-human species recently described in common marmoset, *B. reuteri* and *B. aesculapii*. Group IX (471-250-198-134) was heterogeneous, containing bifidobacterial species of human and non-human origin such as *B. asteroides, B. adolescentis, B. breve, B. kashiwanohense, B. bifidum, B. dentium,* and *B. magnum*. The same for group XI-NP (666-253-133) including *B. gallinarum, B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *suis*, *B. saeculare*, *B. pullorum*, *B. actinocoloniforme, B. biavati*.
Analysing all the currently described species of bifidobacteria, *Taq1* was not able to correctly differentiate the origin of some species in groups II and II-NP (Table 3). Therefore, the 16S rRNA sequences in those groups were further restricted by means of several other enzymes available in the CLC_Sequence Viewer database.

Only enzyme *MaeIII* was able to distinguish between human and non-human borne bifidobacteria, and five homogenous groups were obtained: group X (372-275-185-157-61) including *B. biavatii*, group XI (468-364-157-61) including *B. actinocoloniforme*, group XII (471-405-113-61) containing *B. longum* subsp. *longum* and *B. longum* subsp. *infantis,* group XIII (518-472-61) containing *B. scardovii*, *B. gallinarum*, *B. pullorum* and *B. saeculare*, and group XIV (532-405-113) containing only *B. longum* subsp. *suis* of non-human origin (Table 4).

Table 4. *MaeIII* restriction profiles obtained for each species with information about the pattern attribution and the origin.

Species	Frag1	Frag2	Frag3	Frag4	Frag5	Frag6	*MaeIII* pattern	Origin
B. biavatii	61	157	185	275	372	0	X	Non-human
B. actinocolinoforme	0	61	157	364	468	0	XI	Non-human
B. adolescentis	0	61	114	409	466	0	XII	Human
B. breve	0	61	113	410	466	0	XII	Human
B. dentium	0	61	114	409	466	0	XII	Human
B. faecale	0	61	113	409	467	0	XII	Human
B. bifidum	0	61	114	407	468	0	XII	Human
B. kashiwanohense	0	61	113	407	469	0	XII	Human
B. longum subsp. *longum*	0	61	113	405	471	0	XII	Human
B. longum subsp. *infantis*	0	61	113	405	471	0	XII	Human
B. stellenboschense	0	0	61	461	528	0	XIII	Non-human
B. tsurumiense	0	0	61	466	523	0	XIII	Non-human
B. thermacidophilum subsp. *porcinum*	0	0	61	467	522	0	XIII	Non-human
B. thermophilum	0	0	61	467	522	0	XIII	Non-human
B. thermacidophilum subsp. *suis*	0	0	61	467	522	0	XIII	Non-human
B. thermacidophilum subsp. *thermacidophilum*	0	0	61	467	522	0	XIII	Non-human
B. pullorum	0	0	61	471	518	0	XIII	Non-human
B. scardovii	0	0	61	469	520	0	XIII	Non-human
B. asteroides	0	0	61	470	519	0	XIII	Non-human
B. saeculare	0	0	61	471	518	0	XIII	Non-human
B. stellenboschense	0	0	61	461	528	0	XIII	Non-human
B. gallinarum	0	0	61	472	517	0	XIII	Non-human
B. magnum	0	0	113	416	521	0	XIV	Non-human
B. longum subsp. *suis*	0	0	113	405	532	0	XIV	Non-human

Table 5. Summary of the results obtained from the restriction analysis with the three enzymes.

Species	Origin	*First digestion:AluI pattern*	*Second digestion: TaqI pattern*	*Third digestion:MaeII I pattern*
B. biavatii	Non-human	II	XI-NP	X
B. actinocolinoforme	Non-human	II	XI-NP	XI
B. adolescentis	Human	I	IX	XII
B. kashiwanohense	Human	II	IX	XII
B. dentium	Human	II	IX	XII
B. breve	Human	II	IX	XII
B. bifidum	Human	II	IX	XII
B. faecale	Human	VII	IX	XII
B. longum subsp. *longum*	Human	II-NP	XI-NP	XII
B. longum subsp. *infantis*	Human	II-NP	XI-NP	XII

B. tsurumiense	Non-human	II	IX	XIII
B. asteroids	Non-human	II	IX	XIII
B. thermophilum	Non-human	VII	IX	XIII
B. thermacidophilum subsp. *thermacidophilum*	Non-human	VII	IX	XIII
B. thermacidophilum subsp. *suis*	Non-human	VII	IX	XIII
B. thermacidophilum subsp. *porcinum*	Non-human	VII	IX	XIII
B. stellenboschense	Non-human	I	XI-NP	XIII
B. scardovii	Non-human	II	XI-NP	XIII
B. saeculare	Non-human	II-NP	XI-NP	XIII
B. pullorum	Non-human	II-NP	XI-NP	XIII
B. gallinarum	Non-human	II-NP	XI-NP	XIII
B. magnum	Non-human	II-NP	IX	XIV
B. longum subsp. suis	Non-human	II-NP	XI-NP	XIV
B. cuniculi	Non-human	I	VIII	
B. animalis subsp. lactis	Non-human	I	VIII	
B. animalis subsp. animalis	Non-human	I	VIII	
B. tissieri^{T}	Non-human	II	VIII	
B. tissieri (reference strain)	Non-human	II	VIII	
B. spp. MRM_8.19	Non-human	II	VIII	
B. spp. MRM_9.3	Non-human	II	VIII	
B. pseudolongum subsp. *pseudolongum*	Non-human	II	VIII	
B. pseudolongum subsp. *globosum*	Non-human	II	VIII	
B. choerinum	Non-human	II	VIII	
B. saguini	Non-human	VII	VIII	
B. boum	Non-human	VII	VIII	
B. reuteri	Non-human	I	X-NP	
B. aesculapii	Non-human	I	X-NP	
B. myosotis^{T}	Non-human	I-NP		
B. myosotis (reference strain)	Non-human	I-NP		
B. merycicum	Non-human	III		
B. callitrichos	Non-human	III		
B. angulatum	Non-human	III		
B. eulemuris	Non-human	III-NP		
B. hapalii^{T}	Non-human	III-NP		
B. hapalii (reference strain)	Non-human	III-NP		
B. bombi	Non-human	III-NP		
B. ruminantium	Non-human	IV		
B. bohemicum	Non-human	IV-NP		
B. subtile	Non-human	V		
B. psychraerophilum	Non-human	V		
B. mongoliense	Non-human	V		
B. minimum	Non-human	V		
B. indicum	Non-human	V		
B. crudilactis	Non-human	V		
B. coryneforme	Non-human	V		
B. commune	Non-human	V		
B. lemurum	Non-human	V-NP		
B. pseudocatenulatum	Human	VI		
B. moukalabense	Human	VI		
B. catenulatum	Human	VI		
B. gallicum	Human	VI-NP		

CONCLUSIONS

Bifidobacterium species are characterized by significant host specificity. Based on this hypothesis, Delcenserie et al. (*1*) proposed the use of bifidobacteria as indicators of faecal pollution and developed a PCR-RFLP on the 16S rRNA gene to distinguish the origin (human or non-human) of different bifidobacterial species, testing 64 strains belonging to 13 *Bifidobacterium* species.

In the present study the method described by Delcenserie et al. (*1*) was confirmed in its ability to differentiate between the human or non-human origin of bifidobacterial species. The method uses *AluI* or *Taq1*; we implemented an alternative restriction procedure using MaeIII applied to all bifidobacterial species so far described.

Interestingly *B. angulatum,* till now considered of human origin, clusters in this study with *B. merycicum* and *B. callithricos,* both of non-human origin. This supports the hypothesis that finding a species with only one strain in one single habitat is not sufficient to ascribe this habitat to that species. In fact, this species has been isolated from human faeces, but probably derives from another source. Similarly *B. scardovii*, isolated from female adult patients, viz. a 50-year-old female's blood sample (Sweden), two elderly patients' urine samples (Sweden), and a 44- year-old female patient's hip (*21*), cluster with other non-human species: again the source of this species needs to be revised.

RFLP is a fast and simple strategy to determine the origin (human or non-human) of bifidobacteria, and could be a powerful MST tool for monitoring the source of faecal contamination in the environment.

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5. CONCLUSIONS

Bifidobacteria represent one of the main groups in the human and animal gastrointestinal tract (GIT) and are generally considered host-animal specific bacteria, with demonstrated health promoting properties. The basis of the close relationship between species of *Bifidobacterium* and their hosts is unknown, but it is thought to be due to peculiarities in the bifidobacterial cell-wall structures involved in intestinal epithelium adhesion, or to bifidobacterial ability to metabolize, in the intestine, specific substrates from the host diet. Recently, Endo et al. (2010) observed a richness in term of diversity and abundance of bifidobacteria harboured by two species of New World monkeys. Several molecular approaches to provide an overview of the microbial diversity, mainly based on the polymerase chain reaction (PCR), were developed by taxonomists. And when a definition of their origin, human or animal, is a required, such as in "Microbial Source Tracking" studies as well as in probiotic selection, the RFLP-PCR of 16S rRNA gene sequences was used as rapid tool.

Candidate probiotic strains should satisfy specific features, such as physiology, safety, functional aspects, technological aspects (Saarela et al., 2000). Furthermore, the effect of the probiotic strain on other members of the intestinal microbiota and on the host are to be considered.

Searching for EPSs-producing bacteria is a new challenge in putative probiotic strains selection because EPSs possess beneficial health-promoting effects due to their crucial roles in adhesion mechanisms, control of pathogens, maintaining survival/viability of microorganism during technology food process/storage, and contributing to rheological properties of fermented foods. The ability to produce EPSs has been recognized in different bifidobacterial strains, but a rapid tool for their screening, avoiding the time-consuming EPSs extraction and quantification, are currently unavailable. Folate is a B vitamin with important functions for the health, such as reduction of blood homocysteine levels, formation of red blood cells; it is involved in the protein metabolism, cell growth and division and, in the first months of pregnancy, an adequate folate intake prevents neural tube defects (NTD) and anencephaly. A strain-dependent folate production from bifidobacteria is reported giving an added-value to probiotic strains. Indeed, this is a natural folate form which compared to the synthetic folate, does not cause "masking" of pernicious anaemia.

Bifidobacterial occurrence in non-human primates

The bifidobacterial diversity was explored in several non-human primate subjects to better understand the co-evolution between bifidobacteria and their primate hosts from different evolutionary time-scale. In detail, seventeen subjects selected from Strepsirrhini (*Lemuridae*) such as *Eulemur macaco, Eulemur rubriventer, Hapalemur alaotrensis, Lemur catta,* and from Simiiformes (*Callithrichidae*), such as the New world monkeys *Callithrix jaccus, Pithecia pithecia, Saguinus oedipus* and *Saguinus imperator* and from the old world monkeys *Chlorocebo aethiops* and *Macaca Sylvanus* were

investigated. Cultivable bifidobacteria were detected and isolated from twelve subjects out of seventeen different hosts monkey species: no bifidobacteria were found in *Eulemur rubriventer, Hapalemur alaotrensis, Pithecia pithecia* and the old world monkeys. Notable, the cluster analysis of the bifidobacterial isolates for the recognition of clones, using the program GelCompareII (AppleMath) in order to compare ERIC- and/or BOX- fingerprinting profiles, revealed a richness of unknown strains. The identification was performed with both the *hsp60* RFLP-PCR analysis, and the sequencing of the almost complete 16S rRNA gene sequences. In common marmosets and tamarins, the presence of nineteen novel bifidobacterial species which showed a strong host specific distribution were found together with the five bifidobacterial species previously described by Endo et al. (2012) in *Callithrics jacchus* and in *Saguinus midas* (*Bifidobacterium callitrichos, Bifidobacterium stellenboschense, Bifidobaterium reuteri, Bifidobacterium saguini* and *Bifidobacterium biavati*).

Six novel species, such as *Bifidobacterium aesculapii*, *Bifidobacterium myosotis, Bifidobacterium tissieri, Bifidobacterium hapali*, and other two actually under description, were isolated from 5 baby subjects of the New World Monkey *Callithrix jacchus*. a total of seventeen novel *Bifidobacterium* spp. were also found in an adult subject of *Saguinus oedipus*, and in an adult subject of *Saguinus imperator*. Among these, *Bifidobacterium aeriphilum, Bifidobacterium avesanii* and *Bifidobacterium ramosum* are under description. At last in adult subjects of *Lemur catta* and *Eulemur macaco* the two novel species *Bifidobacterium lemurum* and *Bifidobacterium eulemuris* have been described.

The distribution of microbial communities in non-human primates from eight babies of common marmosets, golden faced saki and Barbary macaques and eleven adult subjects of ring-tail lemurs, black lemurs, red-bellied lemur, Alaotran bamboo lemur, Barbary macaques, grivet, cotton top-tamarin and emperor tamarin, was carried out by using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and rep-PCR. ARDRA was performed with *BamhI*, *HindIII*, *MboI* pair on the amplified universal and the genus-specific for bifidobacteria, 16S rRNA genes. Two fingerprinting techniques, ERIC- and BOX-PCR, were selected for rep-PCR. ARDRA results underlined the potential of the restriction analyses on the bifidobacteria 16S rRNA partial gene sequence, which seems able to distinguish harboured bifidobacteria at the species level. Alternative enzymes should be investigated to improve the method. Getting an overview of the global community diversity, the rep-PCR fingerprinting analysis appears the best technique, even if no additional information about family, genus or species should be made. Cluster analysis on both single and consensus fingerprinting appear to not reflect the phylogenetic history of the host and in some case appear to be affected by the individual diversity. Results about bifidobacteria quantification suggest a low presence in evolutionary old primates, such as lemurs and old world monkey, compared to those in more evolved species, such as tamarins and common marmoset. Additionally, in common marmoset and Barbary macaques the bifidobacteria concentration in adults and baby seems the opposite compared to humans. Contrarily from bifidobacteria, the amount of lactobacilli in faeces of common marmoset not differs in babies and adults, while, for Barbary macaques, lactobacilli and enterobacteria abundance showed the same trend of bifidobacteria increasing from baby to adult subjects.

Coevolution between bifidobacteria and non-human primates

The host-bifidobacteria coevolution of ring-tail lemur, black lemur, common marmoset, cotton top-tamarin and emperor tamarin and respectively related bifidobacteria was studied by different tree-based methods (TreeMap3, Jane4) and global-fit methods (PACo and ParaFit in CopyCat). Not all methods agree that there are extensive cospeciation in this host-bifidobacteria system. The event-based methods did not find significant congruence between tree topologies, probably as a result of occasional host switching by the bifidobacteria and or due to possible failure to speciate events. Nevertheless, the analysis hypothesized a major occurrence of duplication and host-switch events in the system. The global-fit methods statistically support a global co-speciation between host-bifidobacteria, but not all the individual links in the system are significant. All the programs found *Saguinus* spp. and associated bifidobacteria as the main co-evolutionary sub-system with the strongest and statistically significant links.

Selection of potential probiotic bacteria: EPS and folate production

18 isolates from 12 different new bifidobacterial species were described for their phenotypic features. Interesting probiotic traits, such as resistance to extreme condition or extracellular and folate production, were evaluated.

All the strains isolated from primates and tested in this work did not showed haemolytic activity. Suppose their use as probiotic, the ability to survive at the acid condition and at the presence of bile salts in the stomach was evaluated.

The major force driving the choose of a candidate seem the resistance at low pH, indeed, almost all strains showed a good survival after exposure at high concentration of bile salt in the growth medium. Tested strains are able to growth at pH 2.5 while scares or no growth was present at pH 2.0. Notable interesting results were obtained from the tolerance at the high concentration of bile salt (0.75%). Strains belonging to *B. aesculapii, B. myosotis* and to putative new species with type strain MRM_8.19 are the most resistant showing the highest survival rates.

Based on the analysis performed and all the results obtained, soybean milk is a better matrix compared to the low-fat milk. Indeed, all B. aesculapii strains grew very well in soybean milk, producing considerable amounts of EPS, and resulted in high viscosity values.

During the research activity a PCR based method for screening exopolysaccharides (EPSs)-producing bifidobcateria was *in silico* designed and tested, even if *in vivo* analysis are needed to support the proposed technique. Strains belonging to *B. aesculapii* which were positive to the EPSs screening, and which showed a clearly extracellular matrix production characterized as EPSs were further analysed. EPSs from *B. aescualpii* were quantified after growth at different glucose and lactose concentration (1.5-2%). Outcomes suggest an increase in the production at 1.5% of glucose (MRM_3.1 231.61 μg/ml), while low amounts were measured when lactose was present in the

medium. Strains are unable to product significant EPSs in low fat cow milk, whereas high EPSs production was recognized from strain MRM_4.6 (174.50 μg/ml) after soybean milk fermentation. The technological features related to texture and aromatic analysis of fermented soybean and low-fat milks suggest a good viscosity and the production of molecules characterizing yogurts by MRM_4.8, MRM_5.13 and MRM_8.7. Concluding, this work highlights the potential of *B. aesculapii* strains in enhancing aroma and texture of fermented soybean milk due to a high EPSs production.

The production of folate, verified on strains isolated from ring tailed lemur and common marmoset in a folate free medium (FFM), revealed presence of autotrophy for the vitamin only in some strains from common marmoset. All the other strains did not showed growth, requiring an external source of the vitamin. In detail, strains MRM_8.14, belonging to *B. hapali,* and MRM_8.19, belonging to a new bifidobacterial species not already described, are the only folate producer bifidobacteria among tested strains. Moreover, the level of the intracellular folate quantified by HPLC was not high, ranging between 426.13 and 764.94 μg/100g.

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