Alma Mater Studiorum - University of Bologna

Department of Agricoltural Sciences - DipSA

Ph.D. Course in Agricultural, Environmental and Food Science and Technology

Curriculum: Microbial Ecology and Plant Pathology

Cycle XXVI

Settore concorsuale: 07/F2 - Microbiologia Agraria

Settore scientifico disciplinare: AGR/16

Genus *Bifidobacterium*: taxonomy studies and gene expression analysis on folate pathway

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Final Examination Year 2014

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Abstract

Folates (vitamin B9) are essential water soluble vitamins, whose deficiency in humans may contribute to the onset of several diseases, such as anaemia, cancer, cardiovascular diseases, neurological problems as well as defects in embryonic development. Human and other mammals are unable to synthesize *ex novo* folate obtaining it from exogenous sources, via intestinal absorption. Recently the gut microbiota has been identified as an important source of folates and the selection and use of folate producing microorganisms represents an innovative strategy to increase human folate levels.

The aim of this thesis was to gain a fundamental understanding of folate metabolism in *Bifidobacterium adolescentis.* The work was subdivided in three main phases, also aimed to solve different problems encountered working with *Bifidobacterium* strains. First, a new identification method (based on PCR-RFLP of *hsp60* gene) was specifically developed to identify *Bifidobacterium* strains. Secondly, *Bifidobacterium adolescentis* biodiversity was explored in order to recognize representing strains of this species to be screened for their folate production ability. Results showed that this species is characterized by a wide variability and support the idea that a possible new taxonomic re-organization would be required. Finally *B. adolescentis* folate metabolism was studied using a double approach. A quantitative analysis of folate content was complemented by the examination of expression levels of genes involved in folate related pathways. For the normalization process, required to increase the robustness of the qRT-PCR analysis, an appropriate set of reference genes was tested using two different algorithms. Results demonstrate that *B. adolescentis* strains may represent an endogenous source of natural folate and they could be used to fortify fermented dairy products. This bio-fortification strategy presents many advantages for the consumer, providing native folate forms more bio-available, and not implicated in the discussed controversy concerning the safety of high intake of synthetic folic acid.

Key words: Folate, *Bifidobacterium*, *Bifidobacterium adolescentis*, identification, intra-species variability, reference gene evaluation, gene expression studies, HPLC

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Introduction

Folates (vitamin B9) are water soluble vitamins and are essential cofactors for one-carbon transfer reactions in cells. Folate is fundamental for methylation, for the synthesis of nucleic acid and some aminoacids, representing a crucial factor for cellular growth and replication. Folate deficiency in humans may contribute to the onset of several diseases such as anaemia, cancer, cardiovascular disease, neurological problems as well as defects in embryonic development. Human and other mammals are unable to synthesize *ex novo* folate obtaining it from exogenous sources, via intestinal absorption. Many countries have introduced mandatory fortification programs in order to enhance the vitamin status of the populations, with a view to prevent diseases associated with folate deficiency. Fortification programs are carried out by adding folic acid, the synthetic form of folate, during food processing. However an open debate exists regarding the adverse effects of folic acid supplementation, responsible to mask a vitamin B12 deficiency and concerning a possible relationship between cancers and high amounts of folic acid.

Recently the gut microbiota has been identified as an important source of folates. It has been demonstrated that folate produced by gut microbiota can be absorbed in the large intestine, actively contributing to the human folate intake. The use of probiotics able to daily supply natural forms of folate may represent a new healthy biofortification process.

Bifidobacteria are an important group of intestinal microorganisms, known also for their ability to produce vitamins of B group (including vitamin B9). Folate production seems to be a strain-specific characteristic and some bifidobacteria strains can reach high amounts of vitamin content. In addition many factors, as for example the growth medium and the physiological state, can influence the *in vitro* bifidobacteria folate production and composition.

Among fully sequenced species only *B. adolescentis* and *B. dentium* seems to be totally independent in folate production, possessing the entire set of genes involved. The other bifidobacterial species produce folate only in presence of PABA supplementation, a fundamental folate precursor. Moreover *B. adolescentis* strains show the highest amount of folate content, reaching levels similar to yeast

folate production. *B. adolescentis* represents one of the most abundant species of bifidobacteria in the human gut microbiota and its contribution on folate intake could be relevant.

The purpose of this work was to gain a fundamental understanding of folate metabolism in *Bifidobacterium adolescentis.* This specie was chosen as model organism for this work, considering its folate related characteristics that make it a promising species in terms of vitamin B9 production.

Objectives

The purpose of the work reported in this thesis was to gain a fundamental understanding of folate metabolism in *Bifidobacterium adolescentis.* This specie was chosen as model organism for this work, considering its folate related characteristics that make it a promising species in terms of vitamin B9 production.

The work was subdivided in three main phases, also aimed to solve different problems encountered working with *Bifidobacterium* strains. A rapid and correct identification of the *Bifidobacterium* strains under investigation was the first required step of this work, and a new identification method (based on PCR-RFLP of *hsp60* gene) was specifically develop to achieve this goal (PAPER 1 AND 2). Secondly *Bifidobacterium adolescentis* biodiversity was explored in order to identify representing strains of this species to be screened for their folate production ability (PAPER 3). Finally *B. adolescentis* folate metabolism was studied using a double approach (PAPER 4 AND 5). A quantitative analysis of folate content was complemented by the examination of expression levels of genes involved in folate related pathways.

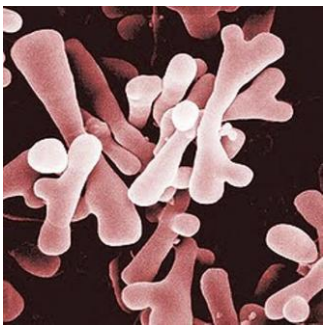
The specific aims of this thesis were:

- the development of an efficient and easy to handle method for *Bifidobacterium* spp. identification (PAPER 1 AND 2)
    - Studies were performed to obtain and validate a new identifying tool based on PCR-RFLP restriction of *hsp60* gene. The method was applied on a total of 37 different *Bifidobacterium* species and was validated analyzing a total of 39 strains belonging to 12 of the most common bifidobacterial species.

- the exploration of the intra-species biodiversity of *Bifidobacterium adolescentis*, (PAPER 3)
    - This study was performed to have a deep insight into *Bifidobacterium adolescentis* genotypic and phenotypic variability. Investigations included a multilocus approach, the use of algorithm for ecotype demarcation and different fingerprinting methods.

- to investigate the *Bifidobacterium* folate metabolism through a double approach: first a quantitative approach, and secondly a transcriptional analysis (PAPER 4 AND 5)
    - In paper 4, we selected and validate putative reference genes for the normalization process required for qRT-PCR
    - Paper 5 was performed to gain a fundamental understanding of folate metabolism in *Bifidobacterium adolescentis*. Several experimental settings were applied in order to monitored folate metabolism in different strains, growth phases and under bile exposure.

Background



Kingdom:	*Bacteria*
Phylum:	*Actinobacteria*
Class:	*Actinobacteria*
Subclass:	*Actinobacteridae*
Order:	*Bifidobacteriales*
Family:	*Bifidobacteriaceae*
Genus:	*Bifidobacterium*

1. *Bifidobacterium*

1.1 History of *Bifidobacterium*

In 1900, Tissier observed and isolated in the feces of breast-fed infants a bacterium with a strange and characteristic Y shape and called it "*Bacillus bifidus*". This bacterium was anaerobic, Gram-positive and did not produce gas during its growth (Tissier H., 1906). He proposed its inclusion in the family *Lactobacillaceae*. For a long time, bifidobacteria were included in the genus *Lactobacillus*. In the 8th edition of Bergey's Manual of Determinative Bacteriology bifidobacteria were classified for the first time in the genus *Bifidobacterium* and comprised eight species (Biavati et al., 2000). Nowadays, according to Taxonomic Outline of the Prokaryotes, the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, sub-class *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. Other genera belonging to this family are: *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia* and *Scardovia* (Biavati and Mattarelli, 2012).

1.2 *Bifidobacterium* physiology and metabolism

Bifidobacteria are Gram-positive polymorphic branched rods that occur singly, in chains or in clumps. They are non-spore forming, non-motile and non-filamentous. Bifidobacteria are ubiquitous, endosymbiotic inhabitants of the gastrointestinal tract and constitute one of the major genera of bacteria that make up the colon microbiota in mammals. They are anaerobic but oxygen sensitivity is a species- and even strain-specific characteristic. Bifidobacteria are chemoorganotrophs, having a fermentative type of metabolism. They produce acid but not gas from a variety of carbohydrates. They are catalase negative (with some exceptions). Their genome GC content varies from 42 mol% to 62

mol% (Biavati and Mattarelli, 2012). The optimum temperature for growth is 37-41 °C, while no growth occurs below 20 °C and above 46 °C. Growth at 45 °C seems to discriminate between animal and human strains. Bifidobacteria are acid-tolerant microorganisms. The optimum pH is between 6.5 and 7.0 and no growth is recorded below pH 4.5. Bifidobacteria are in fact acid tolerant but they are not acidophilic microorganisms. *Bifidobacterium* spp. produce lactic and acetic acid from glucose. The global equation is:

$$2 \text{ glucose} + 5 \text{ ADP} + 5 \text{ P}_i \rightarrow 3 \text{ acetate} + 2 \text{ lactate} + 5 \text{ ATP}$$

This peculiar metabolic pathway is called "fructose-6-phosphate shunt" or "bifidus shunt". The key enzyme of this pathway is fructose-6-phosphate-phosphoketolase, which is considered a taxonomic character for the identification at the genus level (Biavati et al., 2000). Different species produce variable amounts of acetate, lactate ethanol and formate under the same conditions. *Bifidobacteria* utilize a great variety of mono-and disaccharides as carbon sources and are able to metabolize also complex carbohydrates that are normally not digested in the small intestine, their ecological niche. This feature should give an ecological advantage to colonizers of the intestinal environment where complex carbohydrates, such as mucins, are present either because they are produced by the epithelium of the host or because they are introduced through diet.

1.3 Species and ecological niches

The species described to date, can be grouped in four different ecological niches:

- the human intestine, vagina and oral cavity;
- the animal intestine;
- the insect intestine (hindgut of honeybees);
- sewage.

Of the *Bifidobacterium* species found in sewage, five are found also in human, four also in animals and only two are found exclusively in sewage: *B. minimum* and *B. subtile*.

In general, *Bifidobacterium* species are specific either for humans or for animals, with the exception of bifidobacterial species found both in the intestinal microbiota of suckling calves and in breast-fed infants.

At present the genus *Bifidobacterium* hosts 42 species.

*Bifidobacterium actinocoloniiforme, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium animalis (*with two subspecies *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis), Bifidobacterium asteroids, Bifidobacterium biavatii, Bifidobacterium bifidum, Bifidobacterium bohemicum, Bifidobacterium bombi, Bifidobacterium boum, Bifidobacterium breve, Bifidobacterium callitrichos, Bifidobacterium catenulatum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium crudilactis, Bifidobacterium cuniculi, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium indicum, Bifidobacterium inopinatum , Bifidobacterium kashiwanohense, Bifidobacterium longum, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium mongoliense, Bifidobacterium pseudocatenulatum, Bifidobacterium pseudolongum* (with the two subspecies *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum), Bifidobacterium psychraerophilum, Bifidobacterium pullorum, Bifidobacterium reuteri, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium saguini, Bifidobacterium scardovii, Bifidobacterium stellenboschense, Bifidobacterium subtile, Bifidobacterium thermacidophilum* (with the two subspecies *B. thermacidophilum* subsp. *thermacidophilum* and *B. thermacidophilum* subsp. *porcinum), Bifidobacterium thermophilum and Bifidobacterium tsurumiense.*

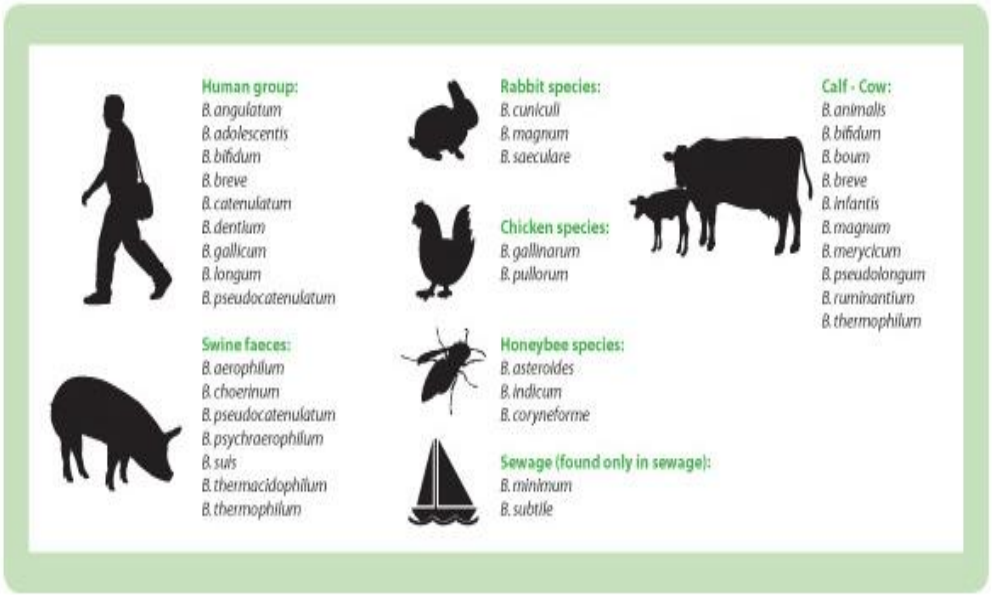


Figure 1: Distribution of principal species of *Bifidobacterium*

Species that inhabit the human body show a different adaptation according to the five different niches colonized: newborn's intestine, adult's intestine, vagina, dental caries and hypochloridric stomach. Numerous studies have demonstrated a bifidobacterial species succession from birth to adulthood. The presence of bifidobacteria in the GI tract of adults and infants has stimulated much interest among bacteriologists and nutritionists. In the intestinal tract of animals and humans, bifidobacteria coexist with a large variety of bacteria, most of which are obligate anaerobes, and they influence each other.

1.4 Bile tolerance and Stress Response in Bifidobacteria

In the human GIT, supplemented bifidobacteria must overcome biological barriers passing through the oral cavity, the stomach and the small intestine, facing daily the bile stress. Bile is a heterogeneous mixture of organic and inorganic compounds (bile salts, phospholipids, cholesterol, bilirubin and proteins) produced by the liver, it is then secreted into the duodenum during the digestion process. Bile exerts an essential physiological role in human health, facilitating the emulsification and absorption of liposoluble nutrients, and exerts a strong antimicrobial activities, contributing to the microbiota balance (Begley et al., 2005). After the delivery of bile into the small intestine, bile salts are absorbed in large part in the ileum, and return to the liver by way of the portal vein, thus completing a portal enterohepatic circulation (Small, 1972). During this process a fraction of bile salts passes into the colon, where it exerts some effects on the local microbiota. As a consequence of their detergent–like properties, bile salts induce membrane damages, influencing the membrane permeability. Additional negative effects of bile regard DNA damage and protein misfolding.

Variation of sugar and amino acid metabolism are indeed directed to contrast the armful actions of these compounds. In this context the ability of bifidobacteria to tolerate bile salts is crucial for their survival in the gastrointestinal environment. To face these stresses, a variety of survival strategies has been especially developed. Bacterial cell surface is the first defense shield against bile effects, for this reason bile exposure influence the expression of bifidobacterial membrane proteins, influencing the fatty acid and phospholipids composition of cell membrane. Bifidobacteria explain other mechanisms to neutralize bile toxicity, such as an active extrusion of bile salts (Piddock, 2006). Guiemonde in a

study on the effects of sub-inhibitory concentration of bile on the expression level of *B. longum*, identified the first bifidobacterial bile efflux pump (BL0920), a multidrug resistance transporters conferring a bile resistance phenotype (Gueimonde et al., 2009). One more mechanism to ensure cell integrity is the production of exocellular polymers, able to create a protective layer against bile effects (Ruas-Madiedo et al., 2005).

In addition to affecting membrane, bile can exert oxidative damage, disturbing macromolecules stability. Bile has been shown to induce aberrant secondary structure in RNA and to induce DNA damage. Bifidobacteria respond with the activation of enzymes involved in DNA repair, moreover bile effect on protein folding is counteracted through a chaperon-mediated response which could preserve the correct protein conformation (Sanchez et al., 2010). Another bile resistant strategy described in bifidobacteria is the bile salts hydrolysis, (Begley et al., 2006) which give rise to a low pH stress on cells, in this case an extrusion of protons is required to maintain the cell balance.

The bifidobacterial response to bile is a strain specific trait and involves also complex cellular processes, with an impact on general metabolic pathways. A recent study has investigated the transcriptional response of bifidobacteria to bile, identifying multiple bile response systems. This work demonstrated that bile exposure was correlated with the over-expression of several genes belonging to different functional categories (Ruiz et al., 2012). Minimal inhibitory concentrations of bile have been described *for Bifidobacterium* species ranging from 0.125% to 2% although differences in experimental conditions make it very difficult to evaluate.

1.5 Probiotic properties of bifidobacteria

The more widely accepted definition for the term "probiotic" is "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/ WHO, 2002). The beneficial modes of action may include: regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function (Salminen et al., 1996), expression of bacteriocins (Mazmanian et al., 2008), enzymatic activity inducing absorption and nutrition (Hooper et al., 2002; Timmerman et al., 2004), immunomodulatory effects (Salzman et al., 2003), inhibition of procarcinogenic enzymes and interference with the ability of pathogens to colonize and infect the mucosa (Gill, 2003). The

probiotic potential is strain-specific. Strains belonging to the same species are unique and may have specific probiotic characteristics (Soccol et al., 2010). Bifidobacteria exert numerous benefits to the host, the most important effects are listed in Table 1.

Beneficial effect	**Reference**
Regulation of the gut microbiota	Quigley, 2010
Production of antimicrobial substances	Martinez et al. (2013)
Alleviation of lactose intolerance	Vonk et al. (2012)
Enhancement of the immune system	Gourbeyre et al. (2011)
Increase bioavailability of bioactive compounds	Rossi et al. (2010)
Production of vitamins	Pompei et al. (2007)
Anticarcinogenic activity	Baffoni et al. (2012)
Prevention of allergic disease	Boyle and Tang (2006)

Table 1: Beneficial effects of *Bifidobacterium* spp.

These beneficial activities constitute an added value in the selection of bifidobacterial strains to be used as probiotics that, besides exerting valuable effects in the regulation of the gut microbiota, may have a role in delivering beneficial compounds to colonic-rectal cells and in exerting protective functions to the host.

1.6 *Bifidobacterium adolescentis* species

Bifidobacterium adolescentis is a Gram-positive, anaerobic bacterium which can be found in several ecological niches. *B. adolescentis* was first described by Reuter in 1963, when this species was found to predominate in the feces of human adults, representing one of the most abundant species of bifidobacteria in human gut microbiota (Yasui et al., 2009).

B. adolescentis species was found also in other ecological niches, namely bovine rumen, human vagina and sewage (Biavati and Mattarelli, 2012). Recent studies pointed out the presence of *B. adolescentis* in additional environments: 14 strains were isolated from Asian elephant feces (Bussenova et al., 2013) and 10 strains from milk of macaque (Killer et al., 2013).

In the last years an increasing interest for *Bifidobacterium adolescentis* has been registered, concerning/ the large probiotic potential of some strains. Several properties has been recently evaluated, for instance Lee (2008) identified the butane extract of B. *adolescentis* SPM0212 as a potent citotoxic agents against tumor cells, by increasing macrophage activations and by inhibiting

tumor cell proliferation. Moreover Cha et al. (2012) demonstrated the antiviral activity of SPM1005-A strain on human papillomavirus type 16, useful for HPV- associated cervical cancer prevention. An et al., (2012) have positively evaluated the efficacy of potential antiviral activities of *B. adolescentis* SPM0214 against herpes simplex virus. Furthermore, the strain SPM0308 showed a strong antagonistic activity against *Propionibacterium acne*, the biological agent of acne vulgaris, significantly reducing the risk of acne development (Lee et al., 2012).

The probiotic potential of *B. adolescentis* strains concerns also another relevant aspect: folate/vitamin production. All the screening available on *Bifidobacterium* spp. and folate content, pointed out/reveal that *B. adolescentis* strains produce the higher levels of B9 vitamins.

This bifidobacterial species is characterized by a large intrinsic variability reflected both in probiotic properties and in all the ecological niches that is able to occupy. Still in 1963, Reuter's studies showed the presence of phenotypic diversity within *B. adolescentis* species, revealing a different fermentation pattern, which induces a subdivision of this taxon into four biovars (called type a, b, c, d) basing on their ability to ferment mannitol and sorbitol and on other serological properties (Reuter, 1963). This phenotypic diversity is even more reflected in a genotypic diversity that emerges from DNA studies (Yasui et al. 2009; Duranti et al. 2013). Yasui provides a first explanation for the intra-species diversity of *B. adolescentis* strains, subdividing the species into two principal groups (called Types I and II), characterized by different content in GC%, variation of cell-surface proteins, and incongruity in genes involved in polysaccharide biosynthesis. In a recent study, Duranti et al. (2013) explored the genomic diversity of seven strains of *B. adolescentis* using a polyphasic approach, involving analysis of 16S rDNA, ITS sequences, pulsed field gel electrophoresis and comparative genomic hybridization. Duranti et al. (2013) demonstrated the existence of extensive variability within *B. adolescentis*. His study highlighted that between 5% and 14% of the open reading frames (ORFs) identified in the *B. adolescentis* ATCC 15703^{T} genome appeared to be absent in the other investigated *B. adolescentis* strains, suggesting a relative rapid diversification of this species (Duranti et al., 2013). The current taxonomic classification seems therefore to be inappropriate to describe the total intra-species diversity that characterizes this group (Yasui et al. 2009; Duranti et al. 2013) and a possible subdivision of this taxon could be taken into account.

2. Folates

2.1 Molecular structure of folates

Folate, also known as folic acid or vitamin B9, is a water soluble vitamin involved in many metabolic pathways. The common term "folates" is used to indicate all the chemical forms of folate, including the different oxidation states and the polyglutamylated forms. The basic molecular structure of folate consists of a pteridine ring conjugated to a para-aminobenzoic acid unit, which is linked to a chain of L-glutamate residue (Figure 3). The pteridine ring can be present in three oxidation state: fully oxidized (folic acid), partially reduced (H_2-folate) or fully reduced (H_4-folate). Only the reduced forms are bioactive. Furthermore at the N-5 and/or N-10 position of the pteridine ring different one carbon units can be present (methyl, formyl, formimino, methylene and methenyl units). The chain of glutamate can be formed by 1 to 11 residues. So, folate exists in a multitude of forms: the total number of folate species is over 100 (Barry Shane, 2010).

Folate can be degraded by factors as light, heath and oxygen exposition. These factors can reduce the activity of the vitamin, changing its chemical structure (Arcot and Shrestha, 2005). Not all the folate species are sensitive in the same way: reduced forms are more susceptible, whereas the oxidized forms present a greater stability. Furthermore natural interconversions occur between different folate species in particular conditions. The general term "folic acid" indicates only the fully oxidized forms, frequently used in the food fortification process, but not present in nature (Blancquaert et al., 2010).

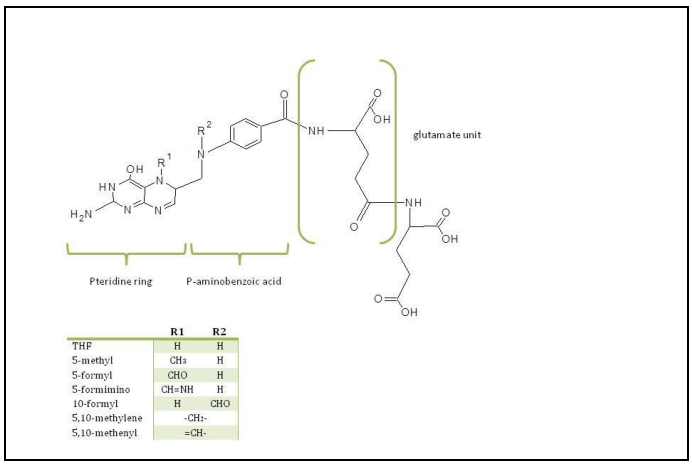


Figure 2: Chemical structure of folates.

2.2 Absorbtion

Dietary folate absorption occurs into the small intestine (Strozzi and Mogna, 2008), where the polyglutamylated forms are hydrolyzed in monoglutamylated forms by folate deconjugase (gamma glutamyl-hydrolase). These monoglutamate forms can be transported through the proximal intestine in two ways: a saturable pH dependent process or via passive diffusion. Other additional amounts of folate, derived from the microbiota activities, are absorbed in the large intestine. Specific carriers are able to mediate the transport of various forms of folate into the enterocytes (Strozzi and Mogna, 2008). After absorptions, folates are converted to $5CH_3H_4$-Folate inside the mucosal cells, then released into the plasma and transported to the liver. At high folate concentrations in the portal vein, this conversion mechanism is saturated: the remaining folate are transformed into H_2-folate by DHFR (Dihydrofolate reductase) (Wright et al., 2005) and further converted in $5CH_3H_4$-Folate. Also this process is saturated at high vitamin concentrations: in this case folates are distributed to other tissues or excreted. Folate so processed is transported to all tissues by the systemic circulation. Within the intracellular environment, folates are conjugated with a one carbon unit, and the polyglutamylated tails build up again. Only the conjugated forms of folate can enter the one-carbon metabolism.

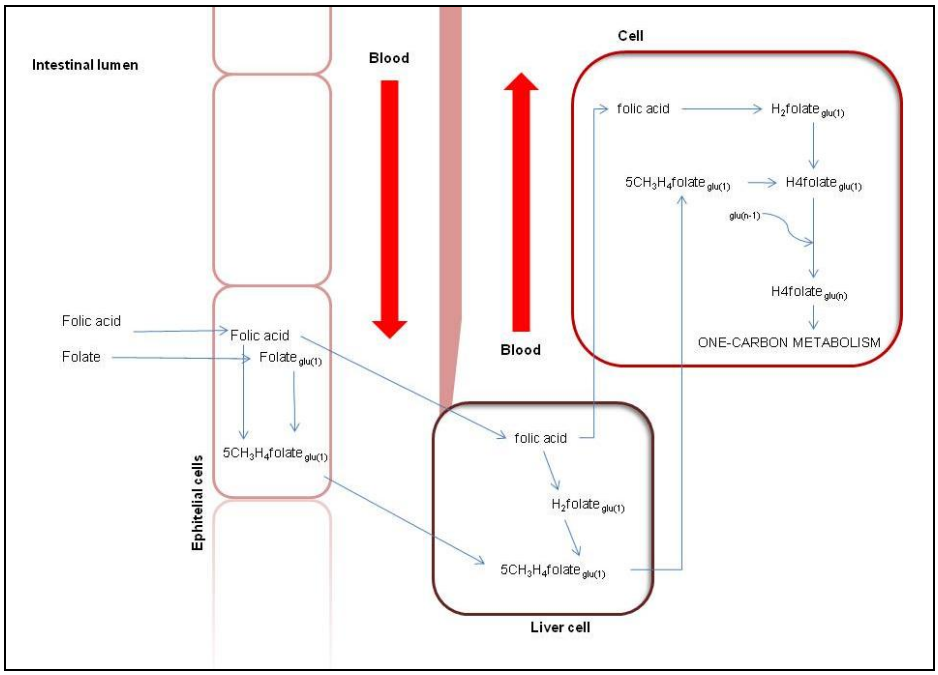


Figure 3: Overview of folate absorption in humans

2.3 Cellular functions

Once inside the cells, folates are involved as cofactors in many different metabolic reactions, implicated in the complex network of reactions of the One Carbon Pool Pathway. Vitamin B9 play a key role in the methyl group biogenesis, in the synthesis of nucleotides, vitamins and some amino acids. Therefore, folate represents an essential compound for the cellular metabolism. Folates are indeed responsible for the biosynthesis of purines, thymidylate, methionine, serine and glycine interconversions and histidine catabolism. Considering its key role in the DNA – RNA biosynthesis cycle and amino acid interconversions, folate is especially important in aiding rapid cell division.

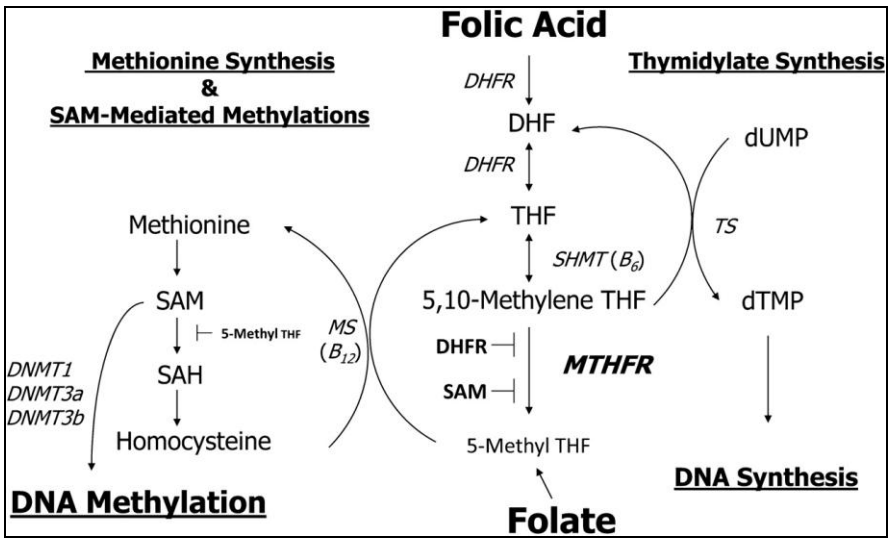


Figure 4: Folic acid metabolism

2.4 Natural sources

Humans and other mammals are unable to synthesize *ex novo* folate, characteristic instead presents in plants, fungi and some bacteria. Mammals obtain folate only from exogenous sources, via intestinal absorption. Folate is naturally present in different food sources. Yeasts, eggs, liver, milk, legumes, green leafy vegetables, cauliflower, broccoli and some fruits like orange are important sources of this vitamin. Fermented food, like yogurt, beer and cheese are additional sources of folate. It's important to underline that many factors can affect the corresponding food folate content: harvest methods, post

harvest treatments, exposure to environmental factors and, of course, the cooking process (Blancquaert et al., 2010). Recently also the gut microbiota has been identified as an important source of this and other vitamins.

2.5 Folate deficiency and disease

Folate intake is suboptimal for most of the world population, even in the rich industrialized countries, where food availability does not represent a problem (Lucock, 2000). Folate deficiency may arise for malnutrition, as well as in case of alcoholism. Malabsorption syndromes, like Chron o celiac diseases, can produce inadequate folate absorption. Another risk factor is the use of some drugs, as an instance oral contraceptives or anti-inflammatories, which can cause folate deprivation. Lacks of this vitamin in humans may contribute to the onset of several diseases such as anaemia, cancer, cardiovascular diseases as well as defects in embryonic development. In addition folate deficiency has been associated to neurological problems, like stroke, depression and Alzheimer disease (Iyer and Tomar, 2009).

ANEMIA

Temporary reduction of dietary intake can produce short term deficiency of folates, which produces characteristic effects on the red blood cells (RBC) parameters. Having RBC a high turnover rate, these cells require high amount of folate, necessary for normal cell division an enzyme synthesis (Fishman et al., 2000). A low folate intake leads to prolongation of the synthesis phase of cell division which results in abnormal red cell precursors, inducing a cell swelling that become megaloblastic (Stover, 2004).

PREGNANCY AND NEURAL TUBE DEFECT

During pregnancy folate requirements increase doubling the need of dietary folates. Indeed pregnancy is associated with an acceleration in carbon transfer reactions, including those required for nucleotide synthesis and cell division. Low maternal folate status is related to increased risk of neural tube defects (NTDs), premature birth and low birth-weight. The most common NTDs are spina bifida and anencephaly (Kibar et al., 2007). Furthermore folate supplementation before conception reduce the frequency of Down's syndrome, cleft palate and preeclampsia (Iyer and Tomar, 2009).

CARDIOVASCULAR DISEASE

Folates exerts several beneficial effects on endothelial function, preventing cardiovascular disease. Low folate intake causes an increase of plasma homocysteine levels, associated to coronary heart disease, being responsible to increase the degree of narrowing of carotid arteries and thus the likelihood of stroke (Wald et al., 2006). Folic acid supplementation can significantly decrease serum homocysteine, suggesting that folates could be useful for the prevention and treatment of cardiovascular disease (Bazzano et al., 2006).

CANCER

Since folate is involved both in nucleotide biosynthesis and in the methylation of various molecules, it has been considered as an active tumor protective agent (Glynn and Albanes, 1994). Several clinical studies suggested that higher folate intake, may significantly reduce the risk of carcinogenesis, reducing the number of DNA aberration. In contrast low folate level can inhibit the growth of tumor, reducing the growth rate of cancer cells (Van Guelpen et al., 2006), and antifolates are therefore used as common anticancer therapy. The role of folate on carcinogenesis is still debated and probably is affected by folate forms, dose and timing of administration (Ulrich and Potter, 2007).

ALZHEIMER'S DISEASE AND COGNITIVE FUNCTIONS

Strong folate deficiency has been correlated with many neurological problem such as depression, schizophrenia and Alzheimer's disease. A daily administration of 5 mg of methylfolate significant improve clinical and social recovery of depressed and schizophrenic patients. Supplementation of vitamin B12, B6 and B9, all involved in homocysteine metabolism, enhances cognitive performances in older adults (Calvaresi and Bryan, 2001). Patients with low folate levels have a higher risk of developing Alzheimer's disease, considering that high levels of homocysteine induce direct neurotoxicity (Mattson and Shea, 2003).

2.6 RDA and fortification programs

The daily recommended intake (RDA) for folate in the European Union is 400 µg/day for adults. This value increases until 600 µg/day in women planning a pregnancy, in order to prevent possible neural tube defects in the fetus, and became 500 µg/day during breastfeeding. Children and adolescents

require lower levels of folate, from 150 µg/day up to 300 µg/day (FAO/ WHO, 2002). Many countries, like Australia, Canada or USA, have introduced fortification programs and enhance the vitamins status of the populations, with a view to prevent diseases associated to folate deficiency. However folic acid fortification may have adverse effects: high folic acid intake may mask a vitamin B12 deficiency, (which can cause anemia and neurological diseases) and seems to increase the risk of developing breast cancer (Blancquaert et al., 2010).

High folate intake (over 1000 µg/day) may reduce the antifolate drugs efficacy, compounds used in the treatment of psoriasis, cancer and arthritis (Blancquaert et al., 2010). In addition there is an open debate regarding the relationship between cancers and high amounts of folic acid (Kim, 2004). For all these controversies some European countries have delayed folic acid fortification program, defined as public health malpractice (Brent and Oakley, 2006). Moreover folic acid (the fully oxidized monoglutamyl form used in the fortification program) is not able to enhance cardiovascular diseases, contrary to what is observed for the natural source of folates. It is noted that natural folate and folic acid have different effects on folate metabolism, so a natural folate biofortification may exclude the negative effect of folic acid fortification (Blancquaert et al., 2010). In this contest, the use of probiotics able to daily supply natural forms of folate may represent a new healthy biofortification process.

3. Folate production by bifidobacteria

Intestinal bacteria represent an important source of folate. It has been demonstrated that folate produced by gut microbiota can be absorbed in the large intestine (Camilo et al., 1996).

Bifidobacteria are an important group of intestinal microorganisms, noted also for their ability to produce vitamins of group B, including folates. Folate production does not seems to be a species-specific characteristic, but it is highly strain dependent. In addition many factors, like growth medium and physiological state, can influence the *in vitro* bifidobacteria folate production and composition. Bifidobacteria folate content can be very high for some strains reaching levels comparable to folate yeast production (*B. catenulatum* ATCC27539 9295 µg/100g DM vs. *S. cerevisiae* CS1 14500 µg/100g DM) (D'Aimmo et al., 2012).

Bifidobacteria show an endogenous production of folate and some forms of this vitamin move through the bacterial cell membrane into the intestinal lumen. Microbes can thus absorb folate from the surroundings or release it in the environment. The distribution of folates in the extracellular environment has been reported in Pompei et al., (2007), using a microbiological assay. Pompei reported that only a small fraction of total folate production, ranged between 9-38%, is stored in the intracellular environment. Therefore folate allocation seems to be mainly extracellular and that represents an important probiotic aspect. It's important to remember that bifidobacteria not only produce, but also consume folate (Lin, 2000). Experimental data available in literature report the balance of these two activities. Folate is essential for methylation, synthesis of nucleic acid and some aminoacids, representing a crucial factor for cellular growth, replication and DNA repair (Crider et al., 2012) (Figure 5).

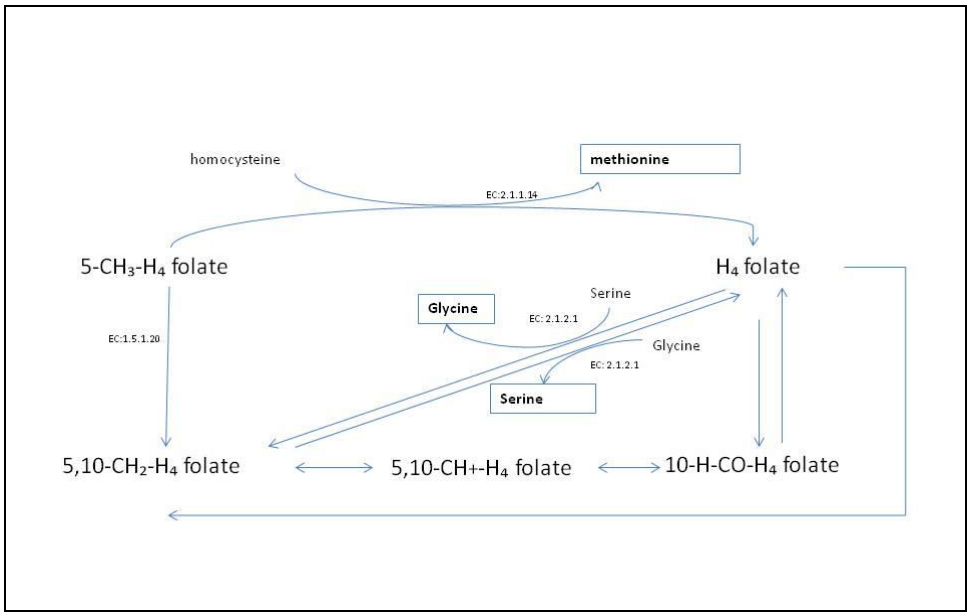


Figure 5: Folate metabolism in *B. adolescentis* ATCC 15703^{T}

3.1 Folate biosynthesis

To synthesize folate bifidobacteria needs to condensate DHPPP (pterin hydroxymethyldihydropteroate) and PABA units (*para*-aminobenzoic acid). DHPPP derived from GTP (Guanosine-triphosphate), and PABA from chorismate. After condensation, a glutamylation step takes place. Compounds are then reduced and a polyglutamyl tail is added to obtain tetrahydrofolate (THF). Using KEGG PATHWAY database it is possible to evaluate the presence of folate key genes in different bifidobacteria genomes sequences available on line (Figure 6) (Kanehisa et al., 2012).

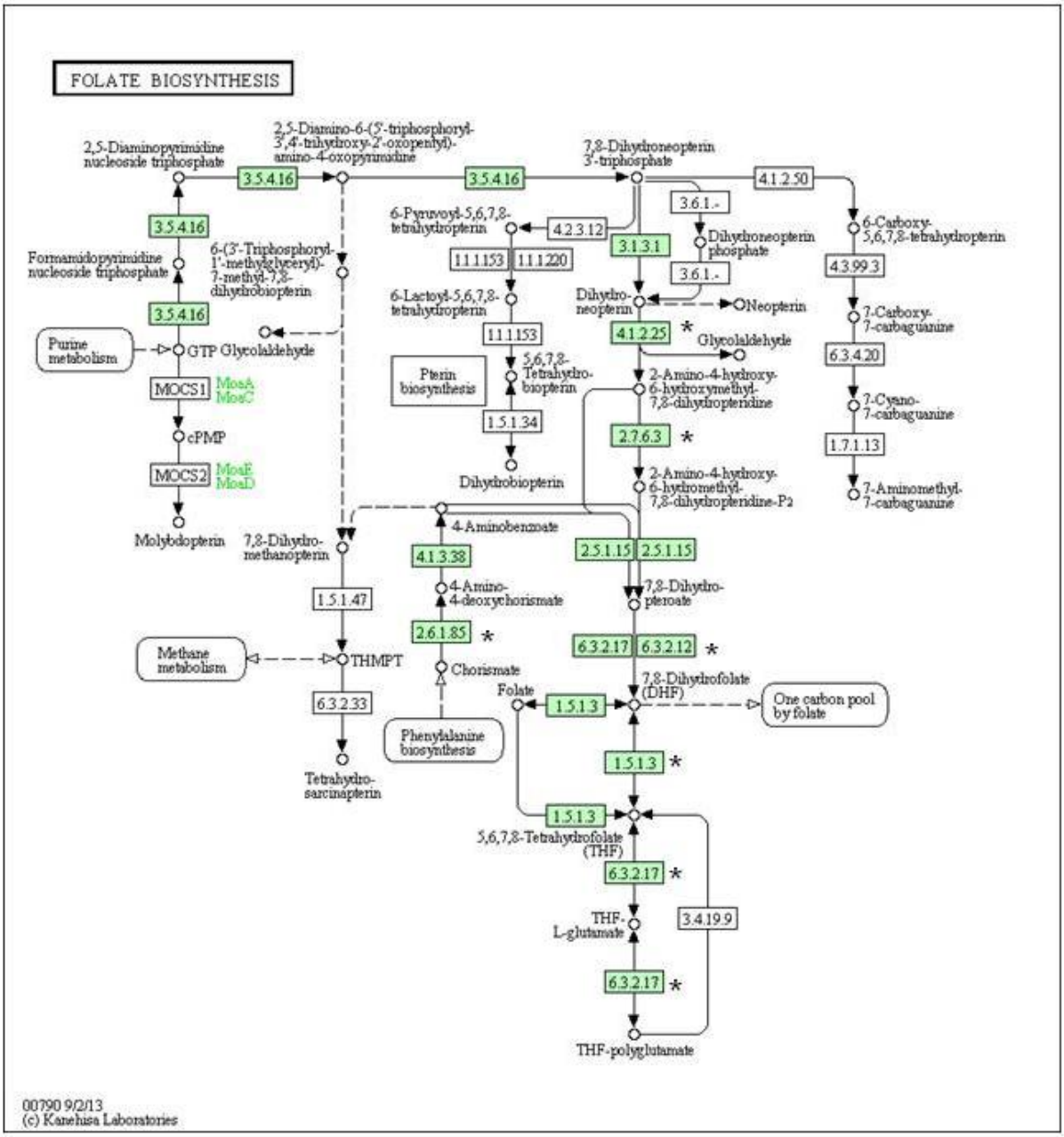


Figure 6: Folate biosynthesis in *B. adolescentis* ATCC 15703. Asterisks indicate genes present in all the sequenced species of Bifidobacterium

The *folC* gene sequence encoding for a polyglutamyl synthetase (or a homologous gene) has been observed in all sequenced microbial genomes (Sybesma et al., 2003). This enzyme is responsible of the polyglutamyl tail elongation, allowing the retention of folate within the cell (LeBlanc et al., 2011). Bifidobacteria seems to have the entire set of genes involved in the shikimate pathway, and are expected to produce chorismate, but not all the species are able to transforms it in PABA. Therefore,

among *Bifidobacterium* genomes, only two sequenced species, *B. adolescentis* and *B. dentium* possess the entire set of genes involved in folate production and seem to be independent in the *de novo* PABA biosynthesis. The other bifidobacterial species seems to be able to produce folate only in presence of PABA supplementation (Rossi et al., 2011) (Figure 7).

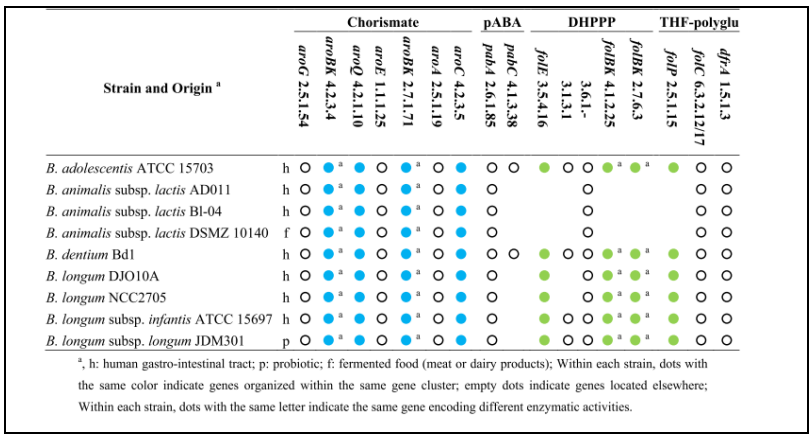


Figure 7: Genes and enzyme for the biosynthesis of chorismate, pABA, DHPP and THF-polyglutamate predicted from the sequenced genomes of genus *Bifidobacterium* (Rossi et al., 2011)

In a screening performed on 76 *Bifidobacterium* strains, it has been demonstrated that in absence of PABA the production of folate was suppressed or reduced for each strain. On the other hands, increasing concentration of PABA led to a great variability in folate production (Pompei et al., 2007). Only for some strains of *B. adolescentis* and *B. pseudocatenulatum* the PABA concentration did not affect the final folate content. In the same study any relation between carbon source and folate production was excluded, as well as the effect of pH. Furthermore in some cases the concentration of this vitamin was really higher in respect to the cells requirements. For this reason it is possible to hypothesize that the biosynthesis is not regulated in some strains.

Regarding *Bifidobacterium* folate composition, the predominant form in the majority of tested strains in D'Aimmo et al., (2012) is $5CH_3H_4$-Folate, followed by H_4-folate. The form HCO-H_4folate was not detected in *Bifidobacterium* spp.

In conclusion, despite the increasing interest regarding folate and microbiota, there are only few studies concerning bifidobacteria and vitamin B9. Furthermore the different experimental settings and methods of analysis in the available studies complicate the interpretation of the results. Many aspects still require to be examined, for example, the degree of glutamate conjugation in *Bifidobacterium* spp. has not been studied: this datum is important to understand the bioavailability of the folate produced by these microorganisms (D'Aimmo et al., 2012). What appears to be increasingly certain, is that bifidobacteria may contribute to the folate requirement of colonic epithelial cells (Leblanc et al., 2007). The high folate levels recently monitored in some *Bifidobacterium* strains have opened new possibilities in their use for increasing folate content in fermented food and dairy products. Furthermore a specific administration of folate-producing probiotics can enhance the vitamin B9 status, preventing folate deficiency. This strategy can avoid the potential risks of fortification with folic acid, supplying elevate levels of natural form of folates, with obviously benefits for the consumers.

Paper 1

Baffoni et al. BMC Microbiology 2013, 13:149
http://www.biomedcentral.com/1471-2180/13/149

BMC Microbiology

METHODOLOGY ARTICLE Open Access

Identification of species belonging to the *Bifidobacterium* genus by PCR-RFLP analysis of a *hsp60* gene fragment

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Abstract

Background: *Bifidobacterium* represents one of the largest genus within the *Actinobacteria*, and includes at present 32 species. These species share a high sequence homology of 16S rDNA and several molecular techniques already applied to discriminate among them give ambiguous results.
The slightly higher variability of the *hsp60* gene sequences with respect to the 16S rRNA sequences offers better opportunities to design or develop molecular assays, allowing identification and differentiation of closely related species. *hsp60* can be considered an excellent additional marker for inferring the taxonomy of the members of *Bifidobacterium* genus.

Results: This work illustrates a simple and cheap molecular tool for the identification of *Bifidobacterium* species. The *hsp60* universal primers were used in a simple PCR procedure for the direct amplification of 590 bp of the *hsp60* sequence. The *in silico* restriction analysis of bifidobacterial *hsp60* partial sequences allowed the identification of a single endonuclease (HaeIII) able to provide different PCR-restriction fragment length polymorphism (RFLP) patterns in the *Bifidobacterium* spp. type strains evaluated. The electrophoretic analyses allowed to confirm the different RFLP patterns.

Conclusions: The developed PCR-RFLP technique resulted in efficient discrimination of the tested species and subspecies and allowed the construction of a dichotomous key in order to differentiate the most widely distributed *Bifidobacterium* species as well as the subspecies belonging to *B. pseudolongum* and *B. animalis*.

Keywords: *Bifidobacterium* spp, *hsp60*, PCR-RFLP, Taxonomy

Background

Members of the genus *Bifidobacterium* are Gram-positive, obligate anaerobic, non-motile, non-spore forming bacteria [1], and are the most important constituents of human and animal intestinal microbiota [2,3]. Recently, news species of bifidobacteria have been described [4-6] and now more than 30 species have been included in this genus.

Bifidobacterium spp. can be detected in various ecological environments, such as intestines of different vertebrates and invertebrates, dairy products, dental caries and sewage. Considering the increasing application of *Bifidobacterium* spp. as protective and probiotic cultures [7-9], and the fast enlargement of the genus, easy identification tools to discriminate new isolates are essential. Moreover, their correct taxonomic identification is of outmost importance for their use as probiotics [2]. Conventional identification and classification of *Bifidobacterium* species have been based on phenotypic and biochemical features, such as cell morphology, carbohydrate fermentation profiles, and polyacrylamide gel electrophoresis analysis of soluble cellular proteins [10]. In the last years several molecular techniques have been proposed in order to identify bifidobacteria. Most available bifidobacterial identification tools are based on 16S rRNA gene sequence analysis, such as ARDRA [11,12], DGGE [13] and PCR

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Table 1 Type-strains investigated

Species	International culture collection
Bifidobacterium adolescentis	ATCC 15703
Bifidobacterium angulatum	ATCC 27535
Bifidobacterium animalis subsp. *animalis*	ATCC 25527
Bifidobacterium animalis subsp. *lactis*	DSM 10140
Bifidobacterium asteroides	ATCC 25910
Bifidobacterium bifidum	ATCC 29521
Bifidobacterium boum	ATCC 27917
Bifidobacterium breve	ATCC 15700
Bifidobacterium catenulatum	ATCC 27539
Bifidobacterium choerinum	ATCC 27686
Bifidobacterium coryneforme	ATCC 25911
Bifidobacterium cuniculi	ATCC 27916
Bifidobacterium dentium	ATCC 27534
Bifidobacterium gallicum	ATCC 49850
Bifidobacterium gallinarum	ATCC 33777
Bifidobacterium indicum	ATCC 25912
Bifidobacterium longum subsp. *longum*	ATCC 15707
Bifidobacterium longum subsp. *infantis*	ATCC 15697
Bifidobacterium longum subsp. *suis*	ATCC 27533
Bifidobacterium minimum	ATCC 27539
Bifidobacterium merycicum	ATCC 49391
Bifidobacterium pseudolongum subsp *pseudolongum*	ATCC 25526
Bifidobacterium pseudolongum subsp. *globosum*	ATCC 25865
Bifidobacterium pseudocatenulatum	ATCC 27919
Bifidobacterium pullorum	ATCC 27685
Bifidobacterium ruminantium	ATCC 49390
Bifidobacterium subtile	ATCC 27537
Bifidobacterium thermacidophilum subsp. *porcinum*	LMG 21689
Bifidobacterium thermacidophilum subsp. *thermacidophilum*	LMG 21395
Bifidobacterium thermophilum	ATCC 25525

with the use of species-specific primers [14-16]. However, 16S rDNA of *Bifidobacterium* spp. has a high similarity, ranging from 87.7 to 99.5% and bifidobacterial closely related species (e.g. *B. catenulatum* and *B. pseudocatenulatum*) or subspecies (e.g. *B. longum* and *B. animalis* subspecies) even possess identical 16S rRNA gene sequences [17,18]. For this reason different molecular approaches have been tested based on repetitive genome sequences amplification, such as ERIC-PCR [19,20], BOX-PCR [21,22] or RAPD fingerprinting analysis [23]. These fingerprinting methods have the disadvantage of a low reproducibility, and they need

Table 2 List of strains investigated to confirm the conservation of RFLP profiles (strains belonging to BUSCoB collection)

Species*	Strain	Source
Bifidobacterium animalis subsp. *animalis*	T169	Rat
Bifidobacterium animalis subsp. *animalis*	T6/1	Rat
Bifidobacterium animalis subsp. *lactis*	P23	Chicken
Bifidobacterium animalis subsp. *lactis*	F439	Sewage
Bifidobacterium animalis subsp. *lactis*	Ra20	Rabbit
Bifidobacterium animalis subsp. *lactis*	Ra18	Rabbit
Bifidobacterium animalis subsp. *lactis*	P32	Chicken
Bifidobacterium bifidum	B1764	Infant
Bifidobacterium bifidum	B2091	Infant
Bifidobacterium bifidum	B7613	Preterm infant
Bifidobacterium bifidum	B2009	Infant
Bifidobacterium bifidum	B2531	Infant
Bifidobacterium breve	B2274	Infant
Bifidobacterium breve	B2150	Infant
Bifidobacterium breve	B8279	Preterm infant
Bifidobacterium breve	B8179	Preterm infant
Bifidobacterium breve	Re1	Infant
Bifidobacterium catenulatum	B1955	Infant
Bifidobacterium catenulatum	B684	Adult
Bifidobacterium catenulatum	B2120	Infant
Bifidobacterium pseudocatenulatum	B1286	Infant
Bifidobacterium pseudocatenulatum	B7003	
Bifidobacterium pseudocatenulatum	B8452	
Bifidobacterium dentium	Chz7	Chimpanzee
Bifidobacterium dentium	Chz15	Chimpanzee
Bifidobacterium longum subsp.*longum*	PCB133	Adult
Bifidobacterium longum subsp. *infantis*	B7740	Preterm infant
Bifidobacterium longum subsp. *infantis*	B7710	Preterm infant
Bifidobacterium longum subsp. *suis*	Su864	Piglet
Bifidobacterium longum subsp. *suis*	Su932	Piglet
Bifidobacterium longum subsp. *suis*	Su905	Piglet
Bifidobacterium longum subsp. *suis*	Su908	Piglet
Bifidobacterium pseudolongum subsp. *pseudolongum*	MB9	Chicken
Bifidobacterium pseudolongum subsp. *pseudolongum*	MB10	Mouse
Bifidobacterium pseudolongum subsp. *pseudolongum*	MB8	Chicken
Bifidobacterium pseudolongum subsp. *globosum*	Ra27	Rabbit
Bifidobacterium pseudolongum subsp. *globosum*	VT366	Calf

Table 2 List of strains investigated to confirm the conservation of RFLP profiles (strains belonging to BUSCoB collection) *(Continued)*

Bifidobacterium pseudolongum subsp. *globosum*	T19	Rat
Bifidobacterium pseudolongum subsp. *globosum*	P113	Chicken

* previously assigned taxonomic identification.

strict standardization of PCR conditions. The use of different polymerases, DNA/primer ratios or different annealing temperatures may lead to a discrepancy in the results obtained in different laboratories [24].

In recent years alternative molecular markers have been proposed for bifidobacteria identification (e.g. *hsp60*, *recA*, *tuf*, *atpD*, *dnaK*) and Ventura et al. [18] developed a multilocus approach, based on sequencing results, for the analysis of bifidobacteria evolution. The *hsp60* gene, coding for a highly conserved 60 kDa heat-shock-protein (a chaperonin), has been evaluated for phylogenetic analysis in bifidobacteria by Jian et al. [25]. The sequence comparison of this gene has been already used for species identification and phylogenetic analysis of other genera (e.g. *Staphylococcus*, *Lactobacillus*) and enteric pathogens [26-28]. A chaperonin database (cpnDB) is available on line, collecting bacterial and eukaryotic sequences (http://www.cpndb.ca/cpnDB/home.php) [29].

Table 3 Expected fragment sizes obtained with *in silico* digestion of the *hsp60* gene sequences

***Bifidobacterium* species**	**GenBank entry**	**Predicted fragment sizes**	**Profile**
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. bifidum	AY004280	22-31-59-181-297	
B. boum	AY004285	22-117-200-251	
B. breve	AF240566	106-139-139-200	
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. 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	17-22-30-32-42-42-109-297	
B. pseudolongum subsp. *globosum*	AF286736	16-17-22-30-32-42-109-323	
B. pullorum	AY004278	16-31-36-42-81-87-297	
B. ruminantium	AF240571	31-106-114-339	
B. subtile	Not available	Not avaiable	+
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	

+ hsp60 sequence of *B. subtile* type strain was not available in the press-time.
† the available sequences at GeneBank and cpnDB belonged to *B. thermacidophilum* (with no distinction in subspecies).
*subspecies not discernable.

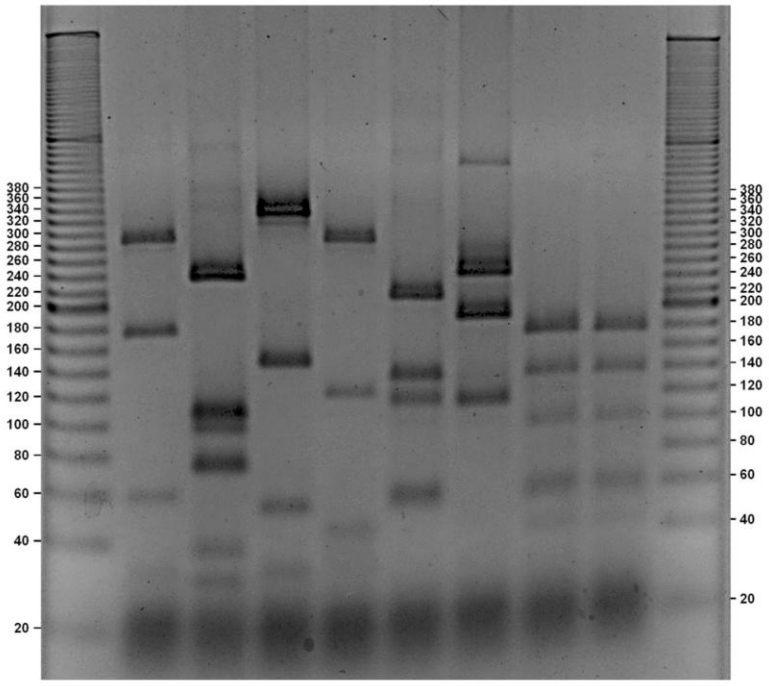

Figure 1 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. bifidum* ATCC 29521; Lane 3, *B. asteroides* ATCC 25910, Lane 4, *B. coryneforme* ATCC 25911; Lane 5, *B. indicum* ATCC 25912; Lane 6, *B. thermophilum* ATCC 25525; Lane 7, *B. boum* ATCC 27917; Lane 8, *B. thermacidophilum* subsp. *porcinum* LMG 21689; Lane 9, *B. thermacidophilum* subsp. *thermacidophilum* LMG 21395; Lane 10, ladder 20 bp (Sigma-Aldrich).

The purpose of this study is the development of a rapid, reproducible and easy-to-handle molecular tool for the identification of *Bifidobacterium* species isolated from various environments. The protocol is based on the restriction endonuclease analysis of the PCR-amplified *hsp60* partial gene sequence (*hsp60* PCR-RFLP) with the use of a single restriction enzyme and has been tested on the 30 most widely distributed *Bifidobacterium* species and subspecies. A diagnostic dichotomous key to speed up profile interpretation has also been proposed.

Methods

Bacterial strains and culture conditions

The type strains used to develop the technique are listed in Table 1, whereas the strains used to validate the method are reported in Table 2. The strains, belonging to BUSCoB (Bologna University Scardovi Collection of Bifidobacteria) collection, were isolated from faeces of human and animals and from sewage. Bacteria were maintained as frozen stocks at −80°C in the presence of skim milk as cryoprotective agent. Working cultures were prepared in TPY medium [1], grown anaerobically at 37°C and harvested at logarithmic phase.

In silico analysis

An *in silico* analysis was performed for the evaluation of a suitable restriction enzyme. Available *hsp60* sequences had been retrieved from cpnDB database and GeneBank, thanks to the work of Jian et al. [25]. *In silico* digestion analysis was carried out on fragments amplified by universal primers H60F-H60R [30] using two on-line free software: webcutter 2.0 (http://rna.lundberg.gu.se/cutter2) and http://insilico.ehu.es/restriction softwares [31]. Blunt end, frequent cutter enzymes that recognize not degenerated sequences have been considered in order to find a suitable enzyme for all the species (e.g. RsaI, HaeIII, AluI, AccII). However *in silico* analysis had been performed also on sticky end enzymes (e.g. AatII, Sau3AI, PvuI).

DNA extraction from pure cultures

10 ml of culture were harvested and washed twice with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6), resuspended in 1 ml TE containing 15 mg lysozyme and incubated at 37°C overnight. Cells were lysed with 3 ml of lysis buffer (100 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2), 220 μl SDS (10% w/v) and 150 μl

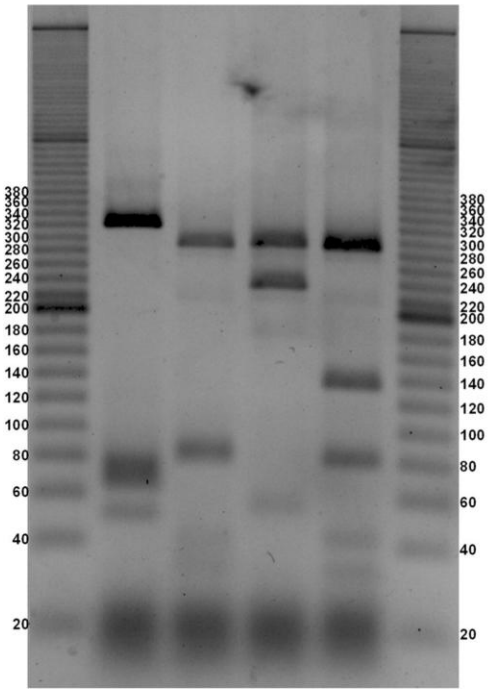

Figure 2 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. minimum* ATCC 27539; Lane 3, *B. pullorum* ATCC 27685, Lane 4, *B. subtile* ATCC 27537; Lane 5, *B. gallinarum* ATCC 33777; Lane 6, ladder 20 bp (Sigma-Aldrich).

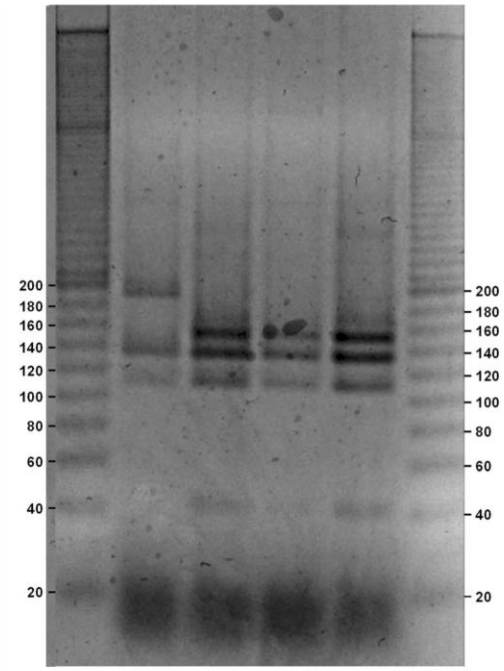

Figure 3 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. breve* ATCC 15700; Lane 3, *B. longum* subsp. *infantis* ATCC 15697; Lane 4, *B. longum* subsp. *longum* ATCC 15707; Lane 5, *B. longum* subsp. *suis* ATCC 27533; Lane 6, ladder 20 bp (Sigma-Aldrich).

proteinase K (>600 mAU/ml, solution) and incubated for 2 hours in water bath at 60°C. One ml of saturated NaCl solution was added and the suspension was gently inverted twice. Pellets were harvested through centrifugation (5000 × g) at room temperature for 15 minutes. After the transfer of clean supernatants in new tubes, DNA was precipitated with 2.5 volumes of cold ethanol (95%) and resuspended in 300 μl of TE buffer [32].

Amplification of gene *hsp60* and restriction with HaeIII

Universal primers were used to amplify approximately 600 bp of the *hsp60* gene in the *Bifidobacterium* spp. investigated. These primers H60F (5'-GG(ATGC)GA(CT)GG(ATGC)AC(ATGC)AC(ATGC)AC(ATGC)GC(ATGC)AC(ATGC)GT-3') and H60R (5'-TC(ATGC)CC(AG)AA(ATGC)CC(ATGC)GG(ATGC)GC(CT)TT(ATGC)AC(ATGC)GC-3') were designed by Rusanganwa et al. [30] on the basis of the conserved protein sequences GDGTTATV and AVKAPGFGD in HSP60. Amplifications were performed in 20 μl volumes with 1.5 μM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 10 μl 2X HotStarTaq Plus Master Mix (Qiagen, Italy) (1,5 mM $MgCl_2$, 1 U Taq, 0.2 mM dNTP, final concentration) and 150 ng/μl DNA. The PCR cycle consisted of an initial denaturation of 5 min at 95°C followed by 35 cycles of denaturation (30s at 94°C), annealing (30s at 61°C) and extension (45 s at 72°C). The PCR was completed with a final elongation of 10 min at 72°C. The PCR amplification was performed with a PCR Verity 96-well thermal cycler (Applied Biosystems, Milan, Italy). After amplification, the product was visualized via agarose gel (1.3% w/v) in 1X TBE buffer and visualized with ethidium bromide under UV light. A 100 bp DNA ladder (Sigma-Aldrich) was used as a DNA molecular weight marker. Bands were excised from agarose gel (Additional file 1: Figure S1) and DNA was eluted with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Germany) in order to avoid possible non-specific amplifications. 3 μl of the eluted DNA was re-amplified in a 30 μl PCR reaction (see above). BSA was added to the reaction (5% v/v, Fermentas). The PCR products (2 μl) were checked for non-specific amplification on agarose gel. 20 μl (~6 μg) of PCR amplicons were digested with HaeIII enzyme. Restriction digestion was carried out for 2 h at 37°C in 30 μl

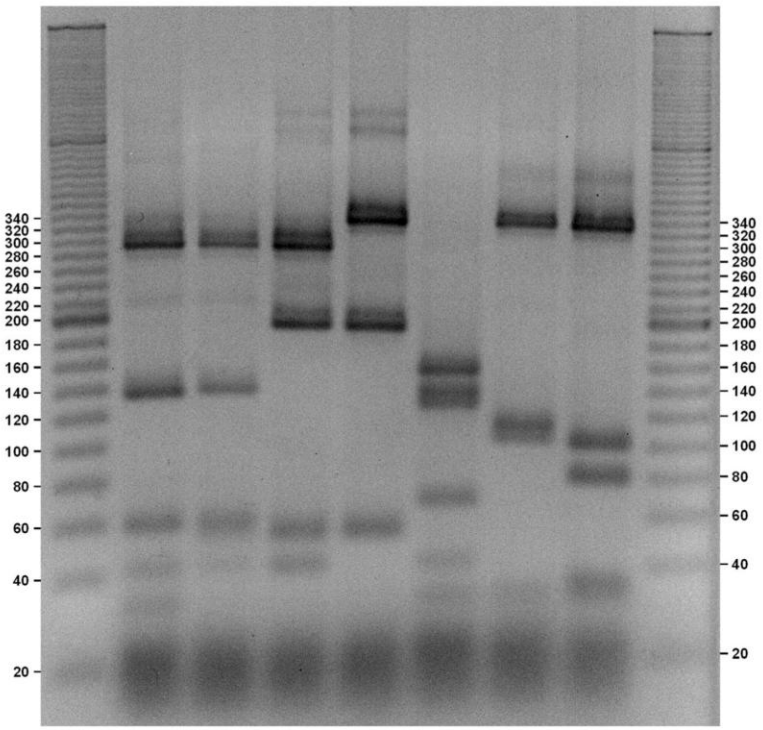

Figure 4 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. merycicum* ATCC 49391; Lane 3, *B. angulatum* ATCC 27535, Lane 4, *B. pseudocatenulatum* ATCC 27919; Lane 5, *B. catenulatum* ATCC 27539; Lane 6, *B. dentium* ATCC 27534; Lane 7, *B. ruminantium* ATCC 49390; Lane 8, *B. adolescentis* ATCC 15703; Lane 9, ladder 20 bp (Sigma-Aldrich).

reaction mixture with 1X SM Restriction Buffer (Sigma-Aldrich), 1.5 μl HaeIII (10 U/μl, Sigma-Aldrich) and water. Digestion products were stained with ethidium bromide and visualized under UV-light (GelDoc™, BioRad), after agarose gel electrophoresis (3.0% agarose (w/v), TBE 1X) at 210 V (3 h). A 20 bp DNA ladder (Sigma-Aldrich) was used. The obtained pictures were elaborated with a free software GNU Image Manipulation Program (Gimp 2.8) only to invert colors and increase contrast.

Precast gradient polyacrylamide gels (4-20%) (Lonza Group Ltd, Switzerland) were also used to obtain RFLP profiles, in order to have a comparison with agarose gels. The vertical electrophoresis apparatus used was P8DS™ Emperor Penguin (Owl, Thermo Scientific) with an adaptor for Lonza precast gels. The run was performed at 100 V in TBE 1X.

Diagnostic key

A dichotomous key was developed comparing *in silico* digestion results and the evaluation of visible bands with the use of ImageLab™ 2.0 software (Bio-Rad Laboratories, Inc.).

Results and discussion

In silico analysis

The analysis and comparison of restriction profiles obtained with *in silico* digestion of bifidobacterial *hsp60* sequences allowed the identification of a set of appropriate frequent-cutter endonucleases that recognize non degenerated sequences. The restriction enzyme HaeIII was found to give the clearest and most discriminatory profiles in theoretical PCR-RFLP patterns, discriminating the majority of *Bifidobacterium* type-strains tested (Table 3). Furthermore, the profiles of other strains, belonging to the investigated species, have been analyzed to confirm the conservation of RFLP profiles within species.

Amplification and restriction analysis of *Bifidobacterium* spp.

Theoretical restriction profiles have been confirmed *in vitro* on agarose gel. The obtained fragments ranged from 16 bp to 339 bp (Table 3). Fragments lower than 25 bp were not considered as they did not help in species discrimination and in addition they co-migrate with

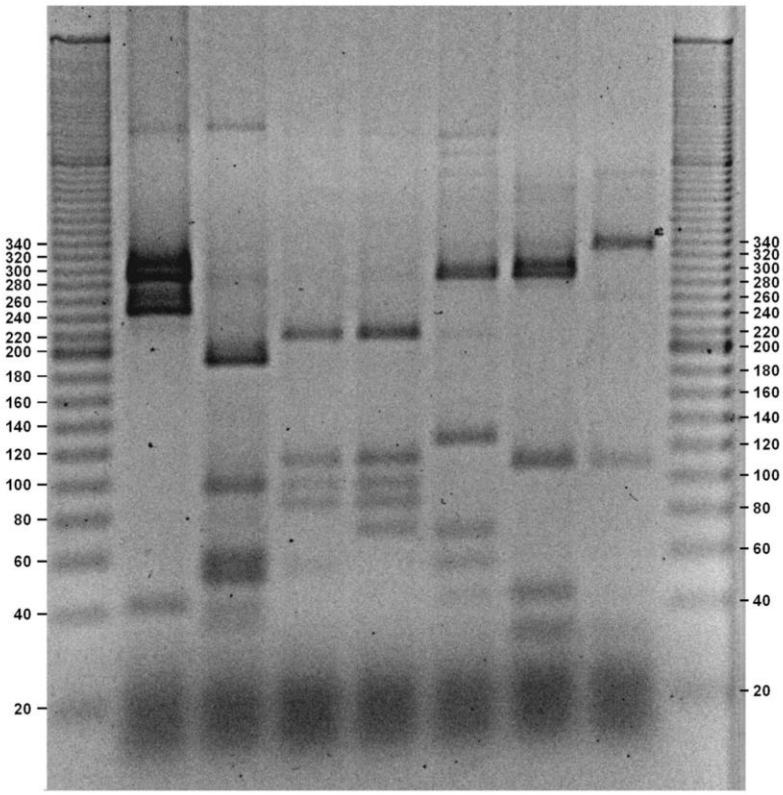

Figure 5 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. gallicum* ATCC 49850; Lane 3, *B. choerinum* ATCC 27686, Lane 4, *B. animalis* subsp. *lactis* DSM 10140; Lane 5, *B. animalis* subsp. *animalis* ATCC 25527; Lane 6, *B. cuniculi* ATCC 27916; Lane 7, *B. pseudolongum* subsp. *pseudolongum* ATCC 25526; Lane 8, *B. pseudolongum* subsp. *globosum* ATCC 25865; Lane 9, ladder 20 bp (Sigma-Aldrich).

primers. Time course analysis of restricted samples showed the formation of a band of ~200 bp in several species due to an over-digestion (data not shown) and this invalidated the RFLP profiles. For this reason the protocol has been optimized at 2 hours restriction time. Fragments greater than 360 bp were also not considered due to a possible incomplete digestion of such long fragments.

The obtained gels (Figures 1, 2, 3, 4 and 5) show species-specific profiles for all type-strains other than *B. longum* and *B. thermacidophilum* subspecies. This technique does not allow the identification of the subspecies belonging to these species, which displayed identical RFLP profiles. Matsuki et al. [14,17] proposed specific primers to differentiate the subspecies of the species *B. longum*, while *B. thermacidophilum* subsp. *porcinum* and *B. thermacidophilum* subsp. *thermacidophilum* can be differentiated according to Zhu et al. [33]. The proposed restriction analysis is efficient in discriminating very closely related species and subspecies as *B. catenulatum*/*B. pseudocatenulatum*, *B. pseudolongum* subsp. *pseudolongum*/*B. pseudolongum* subsp. *globosum* and *B. animalis* subsp. *animalis*/*B. animalis*. subsp. *lactis*.

The same method has been applied with the use of precast gradient polyacrylamide gels. The resolution was greater than that obtained on agarose gels, loading only 4 μl of the restriction reaction instead of the 30 μl used in horizontal electrophoresis. This may allow to reduce the volume of amplification reactions with a consequent reduction of costs.

The comparison between *in silico* digestion and the obtained gel profiles allowed to develop a dichotomous key (Figure 6) for a faster interpretation of the restriction profiles.

Validation of PCR-RFLP analysis on bifidobacterial isolates

39 strains belonging to 12 different species/subspecies (Table 2) have been investigated to validate the PCR-

Figure 6 Dichotomous key to identify species of *Bifidobacterium* based upon HaeIII restriction digestion of ~590 bp of the *hsp60* gene.

RFLP technique. Most of the strains tested were previously identified using biochemical tests and in some cases also molecular techniques (species-specific PCR, 16S rDNA sequencing). The obtained data confirmed a conservation of the profiles concerning the species and subspecies tested. Two figures are available as Additional files (Additional file 2: Figure S2: strains belonging to *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*. Additional file 3: Figure S3: strains belonging to *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *suis*). About 95% of the strains confirmed the taxonomic identification previously assigned. Two strains, B1955 and Su864, previously classified as *B. catenulatum* and *B. longum* subsp. *suis* respectively, gave different profiles from those expected. The RFLP profiles of B1955 turned out to be the same of *B. adolescentis* ATCC 15703 (T), the dichotomous key confirmed the assignment to the *B. adolescentis* species. In addition, Su864 was identified as a *B. breve* strain. These results were also verified through a species-specific PCR [14].

Conclusions

In this work a PCR-RFLP based method to identify *Bifidobacterium* spp. was developed and tested on strains belonging to different species. The technique could efficiently differentiate all the 25 species of *Bifidobacterium* genus and the subspecies belonging to *B. pseudolongum* and *B. animalis*, with the support of an easy-to-handle dichotomous key. The technique turned out to be fast and easy, and presented a potential value for a rapid preliminary identification of bifidobacterial isolates.

Additional files

Additional file 1: Figure S1. Example of agarose gel electrophoresis of hsp60 amplicons from different bifidobacterial strains.

Additional file 2: Figure S2. Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2–6, *B. animalis* subsp.*lactis* strains Ra20, Ra18, F439, P23, P32; Lane 7–8, *B. animalis* subsp. *animalis* strains T169, T6/1; Lane 9, ladder 20 bp (Sigma-Aldrich).

Additional file 3: Figure S3. Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2–4, *B. longum* subsp. *suis* strains Su864, Su908, Su932; Lane 5–6, *B. longum* subsp. *longum* strains PCB133, ATCC 15707 (T); Lane 7–9, *B. longum* subsp. *infantis* strains ATCC 15697 (T), B7740, B7710; Lane 9, ladder 20 bp (Sigma-Aldrich).

Abbreviations

PCR: Polymerase chain reaction; RFLP-PCR: Restriction fragment length polymorphism; HSP60: Heat-shock protein 60; rDNA: Ribosomal DNA; ARDRA: Amplified ribosomal DNA restriction analysis; DGGE: Denaturing gradient gel electrophoresis; ERIC-PCR: Enterobacterial repetitive intergenic consensus-PCR; RAPD: Random amplified polymorphic DNA; cpnDB: Chaperonin database; TPY medium: Tryptone phytone, yeast medium; BUSCoB: (Bologna University Scardovi Collection of Bifidobacteria).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
LB conceived the study. LB, VS and ES carried out all the bioinformatics, RFLP analyses, DNA extractions and culture handling. VS conceived the dichotomous key. MM and PM provided some of the strains tested together with the extracted DNA. DDG and FG supervised the work. LB, VS, DDG and FG contributed to paper writing. All authors read and approved the final manuscript. BB supported the research.

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Received: 20 February 2013 Accepted: 27 May 2013
Published: 1 July 2013

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doi:10.1186/1471-2180-13-149
Cite this article as: Baffoni *et al.*: Identification of species belonging to the *Bifidobacterium* genus by PCR-RFLP analysis of a *hsp60* gene fragment. *BMC Microbiology* 2013 **13**:149.

Paper 2

Anaerobe 26 (2014) 36–40

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Note

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

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ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form
18 December 2013
Accepted 28 December 2013
Available online 4 January 2014

Keywords:
Bifidobacterium
hsp60
PCR-RFLP
Identification

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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http://dx.doi.org/10.1016/j.anaerobe.2013.12.004

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

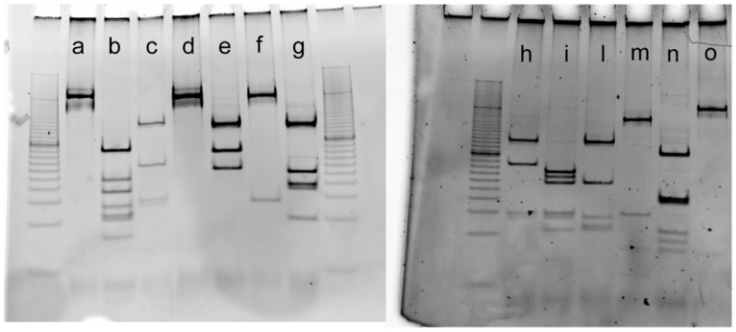
For *B. psychraerophylum* and *B. actinocoloniforme* no RFLP profiles were generated and a single undigested amplicon of 590 bp were obtained, due to absence of cutting sites for *HaeIII* (lanes a and d). In order to correctly identify these 2 species, *hsp60* partial sequencing can be performed. For 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 3

Under submission

Looking inside the intra-species diversity of *Bifidobacterium adolescentis*

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Abstract

Bifidobacterium adolescentis represents one of the most abundant species of bifidobacteria in human health gut microbiota. In recent years, there has been an increasing interest towards strains of this species, due to their relevant probiotic properties such as antibacterial, antiviral and anti-inflammatory activities. *B. adolescentis* strains have been isolated from several ecological habitats, including intestinal tract of humans and animals, human and female of macaque milk, vagina and sewage, suggesting they might have a high adaptability to specific ecological niches. Recently, researchers have highlighted *B. adolescentis* intra-species diversity, exploring the genetic variation of strains from different sources. 20 strains of *B. adolescentis*, isolated from different habitats, were analyzed in this study and genotypic and phenotypic differences were found. The research showed that this species is characterized by an ample variability, highlighted the need for a new re-classification of this group. Moreover the controversial rule of the type strains in realistic species representation was clearly pointed out, since ATCC 15703^T is defined by nonconforming characteristics.

Key Words: *Bifidobacterium adolescentis*, Biodiversity, Ecotype, BOX Rep-PCR, MFA, HMFA, Multilocus approach

1. Introduction

Members of the genus *Bifidobacterium* are high G+C Gram positive, obligate anaerobic, non-motile, non sporeforming bacteria. They are common inhabitants of human and animal body with many species found in the gastrointestinal tract, where represent an important constituent of gut microbiota (Biavati et al., 2012). Bifidobacteria supply several benefits to their host and are largely used as probiotics in food industry and pharmaceuticals. Currently, the *Bifidobacterium* genus holds around 42 species, derived from different sources like human or animal faeces and sewage (Biavati and Mattarelli, 2012). *Bifidobacterium adolescentis* represents one of the most abundant species of bifidobacteria in human gut microbiota (Yasui et al., 2009) and in recent times has been the subject of many studies. The increasing interest for *B. adolescentis* species concerns the large probiotic potential of some strains and recent studies have also highlighted antibacterial and antiviral effects

(Lee et al., 2013), anti-inflammatory activities (Lee et al., 2012) and vitamins production (D'Aimmo et al., 2012).

B. adolescentis was first described by Reuter in 1963, when this species was found to predominate in the feces of human adults. Members of *B. adolescentis* species have been isolated also in other ecological niches (sewage, bowel rumen, infant and animal feces) (Biavati and Mattarelli, 2012), suggesting a large intrinsic variability due to adaptive process (Fraser et al., 2009; Rainey and Travisano, 1998).

In 1963, Reuter's studies showed phenotypic diversity within *B. adolescentis* species, revealing a different fermentation pattern, that induces a subdivision of this taxon into four biovars (namely type a, b, c, d) based on their ability to ferment mannitol and sorbitol and on other serological properties (Reuter, 1963). This phenotypic diversity is even more reflected in a genotypic diversity that emerges from DNA studies (Yasui et al. 2009; Duranti et al. 2013). Yasui provides a first explanation for the intra-species diversity of *B. adolescentis* strains, subdividing the species into two principal groups (called Types I and II), characterized by a different GC% content, variation of cell-surface proteins, and incongruity in genes involved in polysaccharide biosynthesis.

In a recent study, Duranti et al. (2013) explored the genomic diversity of seven strains of *Bifidobacterium adolescentis* by means of a polyphasic approach, involving analysis of 16SrRNA, ITS sequences, pulsed field gel electrophoresis and comparative genomic hybridization. Results from this study (Duranti et al., 2013) demonstrated an extensive variability within *B. adolescentis* and highlighted that between 5% and 14% of the open reading frames (ORFs) identified in the *B. adolescentis* ATCC 15703 genome appeared to be absent in the other investigated *B. adolescentis* strains. These values were higher but similar to those described for other bifidobacteria like *Bifidobacterium longum* subsp. *longum* and should suggest, as also observed in certain enteric bifidobacteria, a relative rapid diversification of genome in this species (Duranti et al., 2013). This identified genomic diversity should be assigned to two classes: (i) mobile DNA that constitutes the *B. adolescentis* mobilome and (ii) plasticity regions of the *B. adolescentis* genome, which may underlie specific adaptations of the investigated strains and which may represent laterally acquired DNA or remnants of ancestral DNA that has not (yet) been lost (Duranti et al., 2013).

Therefore the current taxonomic classification may be inappropriate to describe the total intra-species diversity characterizing this group (Yasui et al. 2009; Duranti et al. 2013) and a possible subdivision of this taxon in other taxonomic units should be taken into account.

However, recent studies have pointed out how the entire concept of prokaryotic species should be reconsidered (Cohan, 2002; Gevers et al., 2005; Koeppel et al., 2008b). In his impressive studies of ecological diversity in microorganisms, Cohen et al. (2002-2008) found that the current methods for

defining prokaryotic species are inadequate to consider the high levels of variability present in nature, and introduced an ecotype concept of species, where members are ecologically similar to one another.

An "ecotype" is defined as ecologically homogeneous clades, whose diversity is constrained by a force of cohesion, periodic selection and genetic drift (Connor et al., 2010). In the last years, many statistical algorithms have been specifically developed to identify ecotype (Ecotype simulator, Adapt ML, BAPS,GMYC) and are currently used to elaborate sequence data. One of that, Ecotype Simulation (ES), provides the most accurate results (Francisco et al., 2012), and for this reason it was taken into account in the present study.

The purpose of this work was to investigate the intra-species diversity within *Bifidobacterium adolescentis,* by means of an ecotype-based approach, modeling the evolutionary dynamics of its bacterial populations. To the best of our knowledge this is the first attempt to identify ecotype in *Bifidobacterium* species. *Bifidobacterium adolescentis* has recently generated growing scientific interest and the genotypic and phenotypic variability characterizing strains of this species should be further investigated.

In the present study 20 strains of *Bifidobacterium adolescentis* isolated from different environments were considered, and their variability was explored.

2. Materials and methods

2.1 Bacterial strains and growth conditions

Type and reference strains (ATCC15703^T, ATCC15704, ATCC15705, ATCC15706) were obtained from the ATCC collection, whereas the others belonged to the BUSCoB (Bologna University Scardovi Collection of Bifidobacteria) collection. The bifidobacteria strains were isolated from different habitat (feces of adults and infant, sewage, feces of orangutan, bowel of rumen). All the information regarding the 21 strains used in this study are listed in Table 1. Cells were cultivate on TPY medium under anaerobic conditions and incubate at 37°C for 24 or 48 hours. Bacteria were maintained as frozen stocks at -80 °C in the presence of skim milk as cryoprotective agent, or freeze dried.

Table 1: List of strains under investigation

Strain	Origin
ATCC 15703	Adult human feces
ATCC 15704 - Re8	Adult human feces
ATCC 15705 - Re9	Adult human feces
ATCC 15706 - Re10	Adult human feces
MB21	Adult human feces
B7304	Adult human feces

B7311	Adult human feces
B7162	Adult human feces
B7283	Adult human feces
B7297	Adult human feces
B7305	Adult human feces
B1955	Infant human feces
F365	Sewage
F200	Sewage
F250	Sewage
ORG 1	Orang feces
ORG 4	Orang feces
ORG 7	Orang feces
ORG 8	Orang feces
Ru424	Rumen of Bowel

2.2 Carbohydrates fermentation

The ability of 20 strains to ferment mannitol/sorbitol were carried out according to procedures described by Crociani et al. (1994). Complex carbohydrates were added to TPY broth (0,5%) and bromcreosol purple (30mg/l) was used as pH indicator (pH 7.5); Bifidobacterial cells from overnight cultures were harvested by centrifugation and resuspended in glucose free-TPY broth to avoid inoculums interference. Growth, variation in final pH and color changes were recorded after 5-7 days of incubation at 37°C in anaerobic jars.

2.3 DNA extraction from pure cultures

DNA of each bacterial strain was extracted according to the method of Rossi et al. (2000).

2.4 PCR-RFLP of *hsp60* gene

Partial hsp60 gene sequences were amplified and restricted according to the method of Baffoni et al. (2013). For two strains (B7340 and F365) partial *hsp60* gene sequence was also cloned using InsTAclone PCR Cloning Kit (Fermentas) and sequenced. All sequencing reactions were performed by Eurofins MWG Operon.

2.5 BOX PCR

The BOX-PCR was performed using the method described in Masco et al 2003 with slight modifications. The primer used in this study is the repetitive sequenced-based oligonucleotide primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'). PCR reactions were carried out in a total volume of 20 µl of reaction mixture containing 10Ul 2X HotStarTaq Plus Master Mix (Qiagen, Italy),

H_2O, 2 µM of BOXA1R (Eurofins MWG Operon, Ebersberg, Germany), and 60 ng of DNA. Amplifications were performed using an Applied Biosystem Verity thermal cycler (Applied Biosystems, Foster City, CA) with the following temperature profiles: an initial denaturation of 5 min at 95°C, followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C) and extension (45 s at 72°C). The cycle was completed with a final elongation of 10 min at 72°C. Aliquots of each amplification reaction mixture (15 µl each) were separated (or resolved) by electrophoresis in 2% (wt/vol) agarose gel containing Sybr safe and visualized under UV light. A 1 Kb DNA ladder (Fermentas) was used as a DNA molecular weight marker (Masco et al 2003).

The BOX-PCR genomic profiles were normalized by Image lab software (BioRad) with a 1-kb DNA ladder as the external reference standard. The BOX-PCR banding patterns of each gel after normalization were acquired by automatic lane and band calling option of Image lab software (BioRad) followed by visual inspection to assure band positions, and manual addition or removal of bands. The resulting data (sample name, molecular weight and peak area of each fragment) were used for cluster analysis in R software, by means of clustering-based peak alignment algorithm developed by *Hishii et al*. (2009).The dendrogram was constructed by using the unweighted pair group method with arithmetic means (UPGMA) tree building method.

2.6 16S rRNA sequencing

In order to verify the taxonomic position 16S rRNA sequencing analysis of all the strains studied was performed. The 16S rRNA gene was amplified by PCR using the primer pair Bif285 (5'-GAGGGTTCGATTCTGGCTCAG-3') and 261 (5'-AAGGAGGTGATCCAGCCGCA-3') (Kim et al., 2011). PCR reactions were carried out in a total volume of 40 µl of reaction mixture containing , 10µl 2X HotStarTaq Plus Master Mix (Qiagen, Italy) , 1µg/µl BSA, 0.2 µM of each primer (Eurofins MWG Operon, Ebersberg, Germany), and 100 ng of DNA. Amplifications were performed using a TGradient thermal cycler (Biometra).The PCR amplification program consisted of an initial denaturation of 5 min at 95°C, followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C) and extension (45 s at 72°C). The cycle was completed with a final elongation of 10 min at 72°C. PCR products were purified using a PCR purification kit (Macherey Nagel) according to the manufacturer's instructions. The purified products were directly sequenced. All sequencing reactions were performed by Eurofins MWG Operon.

2.7 Multilocus approach: *rpoB, dnaJ1, rpoC, purF*

PCR was used to amplify segments of *rpoB, dnaJ1, rpoC, purF.* All the information regarding selected primer pairs are listed in table 2. PCR reactions were carried out in 60 µl of volume containing 10µl 2X HotStarTaq Plus Master Mix (Qiagen, Italy) , 1µg/µl BSA, 0.2 µM of each primer (Eurofins MWG

Operon, Ebersberg, Germany), and 100 ng of DNA. Each PCR cycling profile consisted of a an initial denaturation of 5 min at 95°C, followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C) and extension (45 s at 72°C). The cycle was completed with an elongation phase (10 min at 72°C). PCR products were purified using a PCR purification kit (Macherey Nagel) according to the manufacturer's instructions. The purified products were directly sequenced. All sequencing reactions were performed by Eurofins MWG Operon.

Table 2 Oligonucleotides used in this study

Primer names	Sequence (5'→3')	References
H60_FW	GG(ATGC)GA(CT)GG(ATGC)AC(ATGC)AC(ATGC)AC(ATGC)GC(ATGC)AC(ATGC)GT	Rusanganwa et al. 1992
H60_rev	TC(ATGC)CC(AG)AA(ATGC)CC(ATGC)GG(ATGC)GC(CT)TT(ATGC)AC(ATGC)GC	Rusanganwa et al. 1992
BOXA1R	CTACGGCAAGGCGACGCTGACG	Masco et al. 2003
16S bif 285	GAGGGTTCGATTCTGGCTCAG	Kim et al. 2010
16S 261	GAGGGTTCGATTCTGGCTCAG	Kim et al. 2010
rpoB BifF	TCGATCGGGCACATACGG	Kim et al. 2010
rpoB BifR	CGACCACTTCGGCAACCG	Kim et al. 2010
rpoC-uni	GTGCACTCGGTCCACAG	Ventura et al. 2006
rpoC-rev	CATGCTCAACAACGAGAAG	Ventura et al. 2006
dnaJ1-uni	GAGAAGTTCAAGGACATCTC	Ventura et al. 2006
dnaJ1-rev	GCTTGCCCTTGCCGG	Ventura et al. 2006
purF_uni	CATTCGAACTCCGACACCGA	Ventura et al. 2006
purF_rev	GTGGGGTAGTCGCCGTTG	Ventura et al. 2006

2.8 Sequence analysis

Sequence data were edited using BioEdit software. Sequences were aligned in MEGA 5.05 program (Tamura et al., 2011) using ClustalW algorithms. Alignments were cleaned from problematic alignment blocks using Gblocks 0.91 (Castresana, 2000). Phylogenetic analysis and trees were calculated using MEGA 5.05. Trees were constructed with the Maximum likelihood method and the evolutionary distances were computed using the Tamura Nei model as the substitution model. Bootstrap values of the supertree were computed by re-sampling 1000 times.

2.9 Ecotype simulation

Ecotype simulation (ES) is an algorithm, which quantifies the ecological diversity within a community by analyzing its evolutionary history as result of net ecotype formation and periodic selection. ES models an ecotype as a population genetically cohesive and ecologically distinct to other group, without the need for inputting any ecological data (Koeppel et al., 2008c). The program employs an algorithm for the estimation of the rates of periodic selection and drift, the net rate of ecotype formation, and the number of ecotypes, so as to yield a clade sequence diversity pattern with maximum likelihood. Individual ecotypes are demarcated by determining the largest sub-clades that

are each consistent with containing a single ecotype (Cohan 2009). An ecotype has been defined as "a paraphyletic or monophyletic group of close relatives that are ecologically interchangeable, in that the members of an ecotype share genetic adaptations to a particular set of habitats, resources and conditions, and different ecotypes are distinct in their ecological adaptations". ES gives an ecological interpretation of a sequence based phylogeny. The program requires concatenate sequences as input data, thus the partial sequences of housekeeping genes previously obtained were aligned using ClustalW multiple alignments. After the alignment, all gaps were checked in the chromatograms and removed. Sequence alignments were analyzed using Ecotype Simulation (ES) to estimate the number of putative ecotypes and the rates of ecotype formation (Koeppel et al., 2008c) using the option of 1.5 precision match between observed and simulated data. Different strains were considered belonging to the same ecotype when they presented a confident interval of 1, it means that the possibility that those strains represent a single ecotype is within the 95% confidence range (Koeppel et al., 2008a).

2.10 Nucleotide accession numbers

The GenBank accession numbers for the partial *16S rRNA, hsp60, rpoB, dnaJ1, purF, rpoC* gene sequences used and generated in this study are listed in Additional File 1.

2.11 Statistical analyses

Multiple Factor Analysis (MFA) is a factor analysis method focusing on mixed data composed of sets of quantitative, categorical and frequency variables (Escofier and Pagès, 1994).

All the variables taken into account in the present study were considered for MFA analysis: fermentation profiles, *hsp60* PCR-RFLP profiles, BOX- analysis and gene sequences. The Ecotype groups were indeed excluded in order to prevent possible influences in cluster configuration, because they are based on gene concatenation information, already included in the elaboration. Multiple Factor Analysis (MFA) was performed using FactoMineR (Lê et al., 2008), an R package for Multivariate Analysis (R Development Core Team, 2005). Hierarchical Multiple-Factor Analysis (HMFA) was performed for classifying strains based on phenotypic and genetic data.

3. Results

20 different strains of *Bifidobacterium adolescentis* were selected and studied. Most of them derived from BUSCoB collection (Bologna University Scardovi Collection of Bifidobacteria, Bologna, Italy) but not all were identified using the same method (metabolic activities, DDH analyses). Therefore, the correct assignment of these strains to *Bifidobacterium adolescentis* species was firstly confirmed by means of *hsp60* PCR-RFLP analysis (Baffoni et al 2013).

3.1 PCR-RFLP of *hsp60* gene

The technique described in Baffoni et al. (2013) allows a rapid classification of all tested *Bifidobacterium* species other than *B. longum* and *B. thermacidophilum* subspecies, which display identical RFLP profiles.

The 20 *Bifidobacterium adolescentis* strains under investigation were tested with this method. All the strains of *B. adolescentis* derived from ATCC revealed an identical restriction profile, whereas others were found showing two new banding profiles, not associable with any other pattern previously obtained and published in Baffoni et al (2013) and Stenico et al. (2014).(Baffoni et al., 2013)(Baffoni et al., 2013)(Baffoni et al., 2013)(Baffoni et al., 2013)(Baffoni et al., 2013) Fifteen strains showed a RFLP pattern which we indicated as profile b (table 3 and figure 1), only the strain F365 revealed a third pattern profile c. A comparison of the three different restriction profiles is shown in Figure 1.

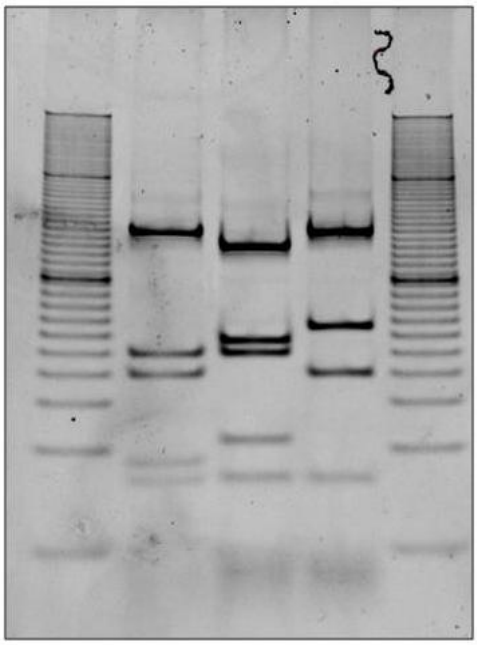

Figure 2: Acrillamide gel electrophoresis of digested hsp60 DNA fragments with *haeIII* (negative image)

Hsp60 amplicon from strain B7304 was cloned and sequenced as representative for the new banding profile b. Despite several unsuccessful efforts using different primer pairs and PCR settings, *hsp60* sequence could not be obtained for F365 strain (profile c), but further analyses are in progress. The B7304 sequence was then analyzed *in-silico* using WebCutter2.0 and the theoretical RFLP profile confirmed (see table 3) with those obtained *in vitro*.

Table 3: *in-silico* pattern of hsp60 gene fragment

Strain	Restriction pattern	Profiles
ATCC 15703	a	31-36-81-103-339
B7304	b	32-42-110-117-296
F365	c	c.a 32-80-140-339

The level of partial *hsp60* gene sequences similarity of the strains B7304 and ATCC 15703 was calculate using BLASTn (Altschul, 1997), and reveal a 95% of homology. This value should suggest inter-species or inter-subspecies similarity rather than intra-species homology, as proposed by Zhu et al. (2003).

3.2 16S rRNA sequencing

As a consequence of PCR RFLP results, with the aim to better characterize the strains analyzed, partial *16S rDNA* sequence analysis was carried out. Each obtained sequence was identified by a BLASTN search against the EzTaxon-e database, using hierarchical taxonomic information (Kim et al., 2012) and robust pairwise global sequence alignment. Comparative sequence analysis with the databases revealed that all fragments had high similarity (ranging from 98% to 99,72%) to the *16S rRNA* sequence of strain ATCC 15703 (Table 4). All these values were above the suggested cutoff criteria for species definition (Tindall et al., 2010). It is also to be highlighted that *16S rRNA* gene sequence of Ru424 strain showed relatively high similarity (99%) also with *B. stercoris* JCM 15978^T sequence. This apparent inconsistency can be explained by a recent study (Killer et al., 2013) that propose a reclassification of *B. stercoris* as a later heterotypic synonym of *B. adolescentis*. All the sequences were subsequently aligned using ClustalW in MEGA 5.1 program (Tamura et al., 2011) and a phylogenetic tree was build on the basis of partial *16S rRNA* using Maximum Likelihood (Fig. 1).

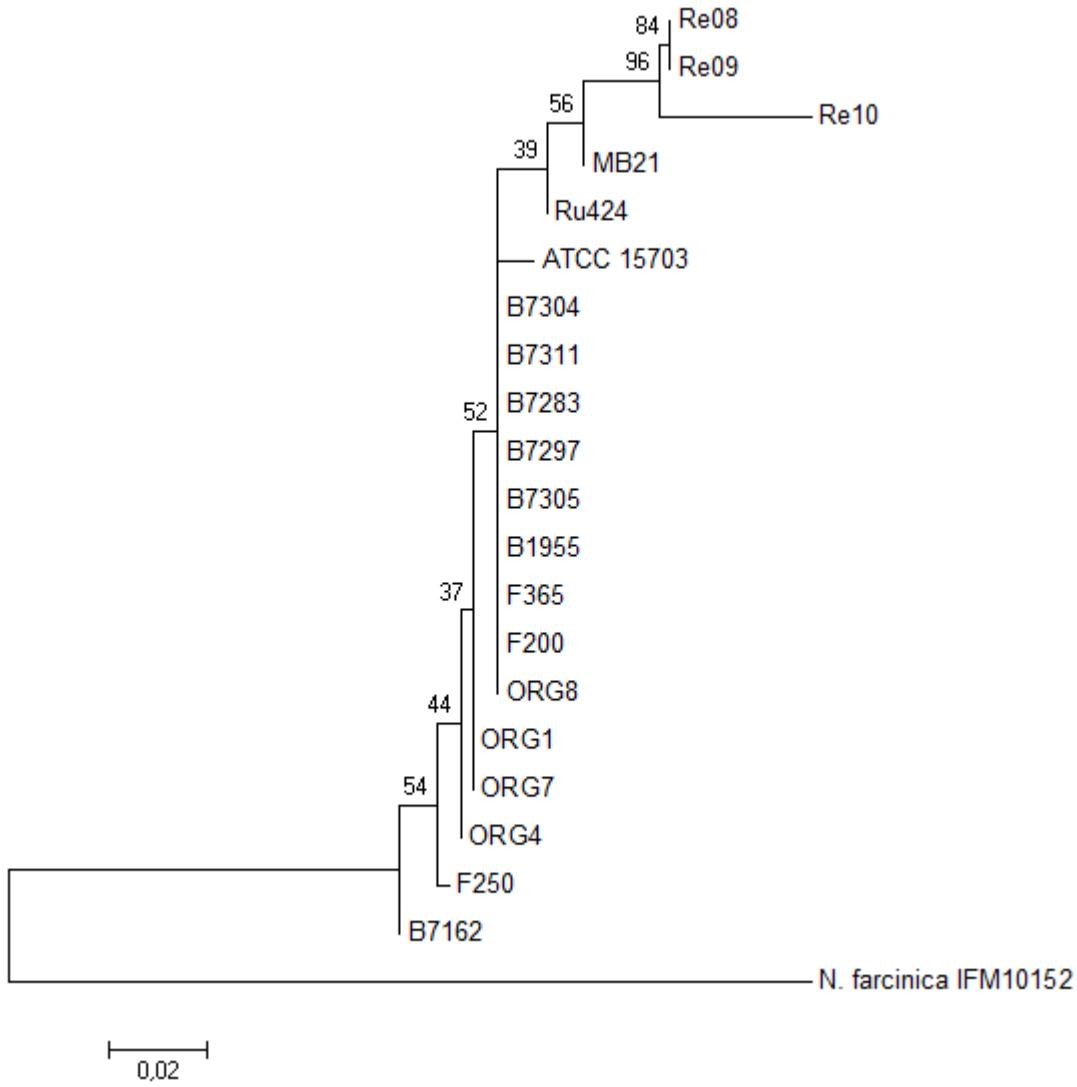

Figure 2: Phylogenetic tree of *Bifidobacterium adolescentis* strains used in this study, computed on the basis of 16S rRNA gene sequences. Bootstrap values were reported for a total of 1000 replicates. Tree was calculated by the Maximum Likelihood method and Tamura model. The tree was rooted using *Nocardia farcinica* IFM 10152.

3.3 Multilocus analysis

Despite most available bifidobacterial identification tools are based on *16S rRNA* gene sequence analysis, a multigenic approach is more suitable in order to assess their relative taxonomic position. Indeed, bifidobacteria share a high degree of similarity or even possess identical *16S rRNA* gene sequences, and different molecular markers should be used for discriminating at subspecies level or for investigating intraspecies biodiversity. For this reason multilocus approach is currently considered a stronger tool for the analysis of bifidobacteria evolution (Ventura et al., 2006). Based on the above, we obtained the partial sequence of 4 genes, *rpoB, purF, dnaJ1, rpoC* for each strain considered in

this study. The selected housekeeping genes belong to different functional categories, and their enormous value as tool for bifidobacterial identification has been already demonstrated in several study (Kim et al., 2010; Ventura et al., 2006).

Using a concatenation of these molecular marker (*dnaJ1, rpoC, rpoB, purF*) it was possible to explore the intra-species variability of *Bifidobacterium adolescentis*. A phylogenetic tree was build basis on genes sequences concatenation (Figure 3), and in addition other four phylogenetic trees were produced using the individual genes (data not shown). The discriminatory power of the concatenated tree was much more significant than that observed with each single gene. Moreover, concatenation allowed an increase in deep-node bootstrap values and thus led to a considerable increase in tree robustness: in the concatenation tree, 73.7% of the nodes were supported by bootstrap values greater than 80%.

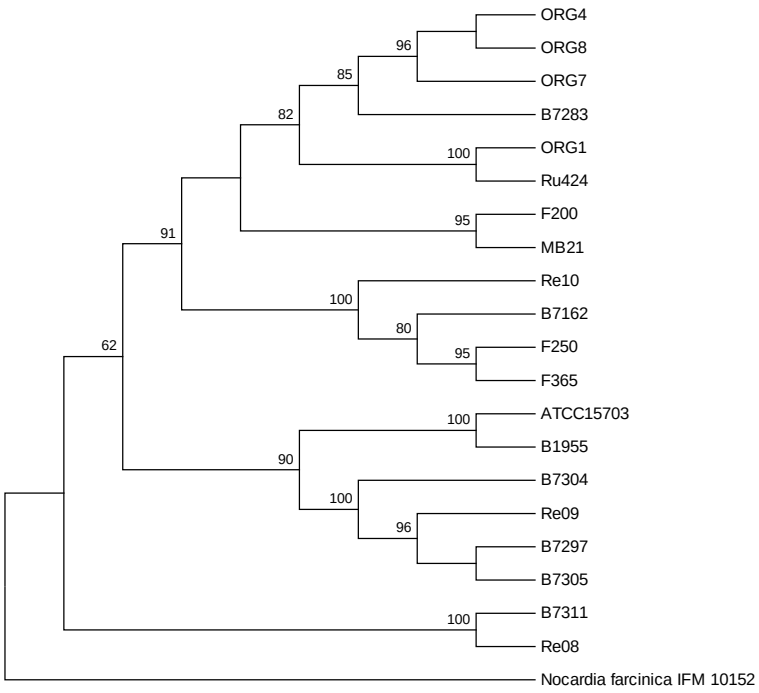

Figure 3: Phylogenetic tree of the species *Bifidobacterium adolescentis* computed from the concatenation of *purF, rpoC, rpoB, dnaJ1* gene sequences by the Maximum Likelihood methods and Tamura model. Bootstrap values were reported for a total of 1000 replicates. The tree was rooted using *Nocardia farcinica* IFM 10152

3.4 Rep-PCR fingerprinting

Rep-PCR fingerprinting using the BOXA1R is a powerful tool, used in taxonomic framework also for *Bifidobacteria* identification. Because of its highly discriminatory power, Rep-PCR fingerprinting using the BOXA1R allows the identification at the subspecies level (Masco et al. 2003).

All the strains of *Bifidobacterium adolescentis* were subjected to BOX-PCR fingerprinting. All the reactions were performed in a single PCR run in order to ensure a more accurate analysis of the profiles, since banding patterns are always affected by the lack of repeatability.

For the cluster analysis, data matrixes obtained from BOX-profiles detection with ImageLab software were used as input, in the complete-linkage clustering algorithm (Ishii et al., 2009), based on R language (R Development Core Team, 2005), which represents a valid tool for analyzing various DNA fingerprints, such as rep-PCR data. Analysis results are represented as a dendrogram; different strains were grouped in the same cluster when an *au p*-value higher than 99% was detected and they were graphically enclosed in a rectangle.

The algorithms identified 4 robust clusters (called A, B, C, D), whereas some strains cannot be included in any groups. Cluster A is a solid group, including all the Orangutan strains (ORG1, ORG4 ORG7 and ORG8), F365, Ru424, B7311 and B7304. Cluster B included 3 strains isolated from adult human feces (B7305, B7297, B7283) and was closely related to cluster C, which was constituted by F250 and B7162. The strain F200 occupied a distinct subline branching at the periphery of the cluster C. Re08 and Re09 building the cluster D. The remaining 4 strains (B1955, ATCC 15703, MB21 and Re10) were dispersed among the phylogenetic tree, establish two unstable clades. Considering the ability of BOXA1R to identify strains at subspecies levels, the presence of 4 groups in this cluster analysis, could reflect the wide variability of *B. adolescentis* species.

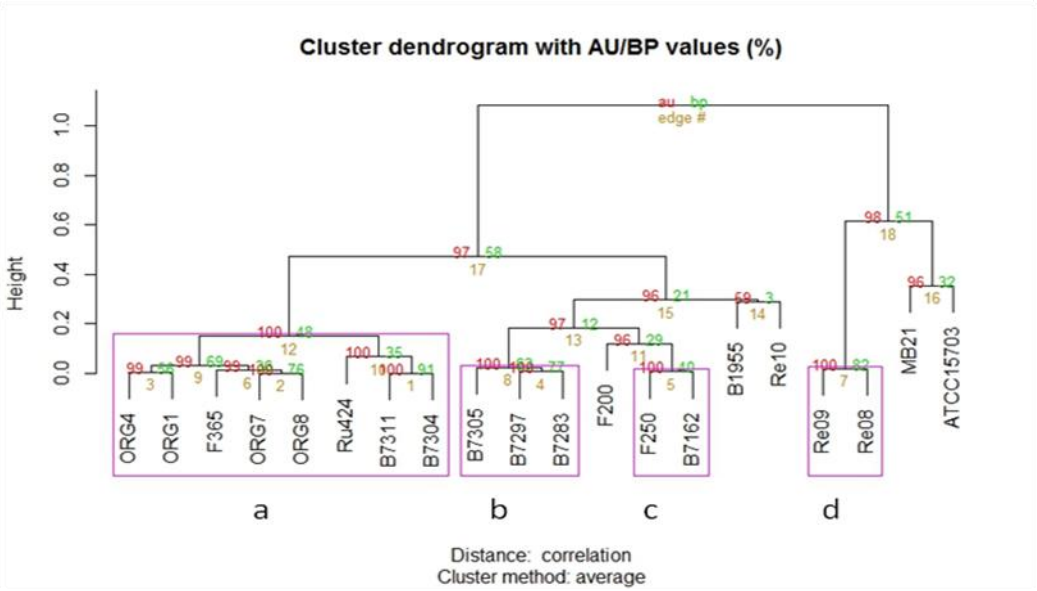

Figure 4: Dendrogram generated after cluster analysis of digitized BOX-PCR fingerprinting of strains. Rectangles indicate clusters (p-values of 0.01). Au is the "approximately unbiased" p-value calculated by multiscale bootstrap resampling (1000 replications); BP represents the "bootstrap probability" value and edge indicates the clusters.

3.5 Mannitol/Sorbitol fermentations

The fermentation of these two specific carbohydrates represents a key for the biovars identification of *B. adolescentis*. Based on their ability to ferment mannitol and sorbitol, 10 strains were identified as biovars B (mannitol+/sorbitol-), other 11 strains were collocated in biovars B (mannitol-/sorbitol+). Re09 as biovars d and ATCC 15703 as biovars A (biovars A +/+ ; biovars D -/-) Information regarding carbohydrates fermentation are listed in Table 2.

Table 4: List of strains investigate and relative information regarding fermentation pattern, PCR-RFLP profile and % identity of 16S rDNA

Strain	Reuters type	PCR-RFLP profile hsp60	% identity 16S rDNA
ATCC 15703	**a**	profile a	100%
Re8	**b**	profile a	98%
Re9	**d**	profile a	98%
Re10	**c**	profile a	98%
MB21	**b**	profile a	99,72%
B7304	**c**	profile b	98,12%
B7311	**c**	profile b	99,55%
B7162	**c**	profile b	98,10%
B7283	**c**	profile b	99,52%
B7297	**c**	profile b	99,52%
B7305	**c**	profile b	99%
B1955	**c**	profile a	99,45%
F365	**c**	profile c	99,46%
F200	**c**	profile a	99%
F250	**c**	profile b	98,68%
ORG 1	**b**	profile b	99,30%
ORG 4	**b**	profile b	99,05%
ORG 7	**b**	profile b	99,18%
ORG 8	**b**	profile b	99,50%
Ru424	**b**	profile a	99%

3.6 Ecotype simulation

Ecotype simulation (ES) is an algorithm, which quantifies the ecological diversity within a community by analyzing its evolutionary history as result of net ecotype formation and periodic selection. ES was performed on different molecular markers concatenated sequences (housekeeping genes *rpoB, dnaJ1, rpoC* and *purF)* belonging to the studied *Bifidobacterium adolescentis* strains. The phylogeny of the *Bifidobacterium adolescentis* clade rooted by *Nocardia farcinica.* ES of the history of the

concatenated gene sequence in *Bifidobacterium adolescentis* estimated the presence of 7 putative ecotypes (Figure 5). The predicted sigma rates (rate of periodic selection) were higher (0.067) than predicted omega rates (rate of ecotype formation) (0.003), suggesting that ecotype formation events are relatively rare and that putative ecotypes are regularly constrained by periodic selection.

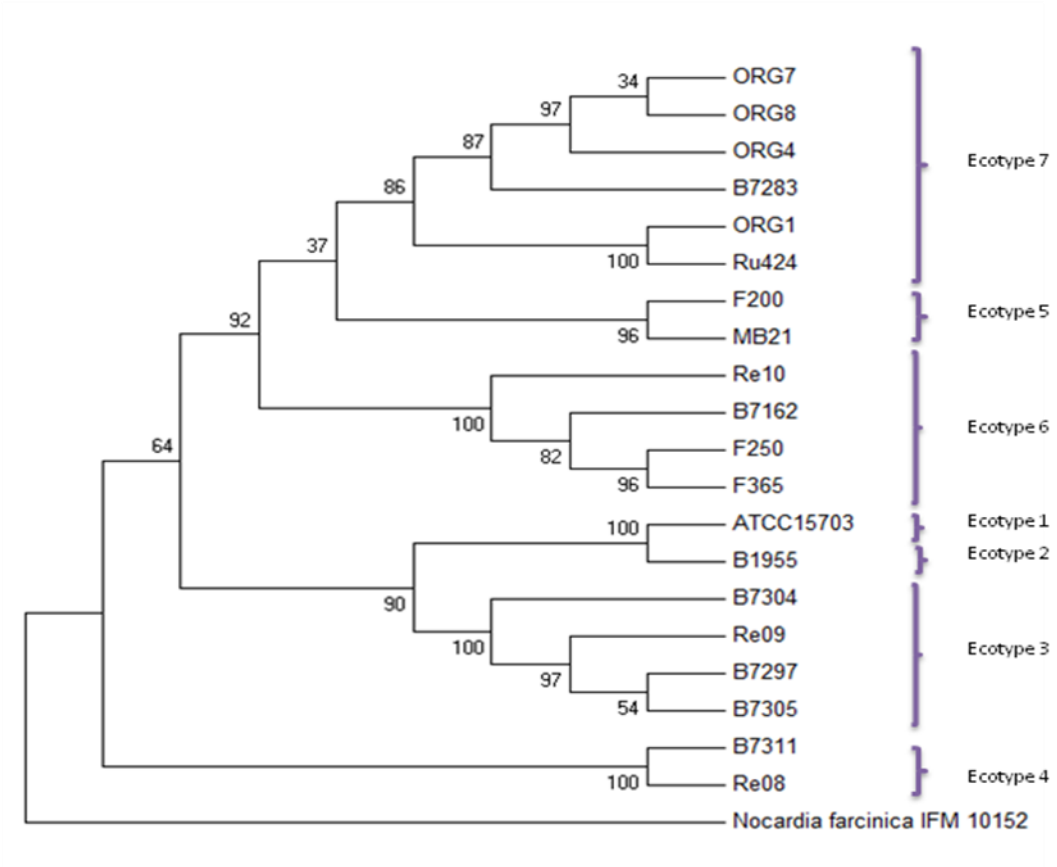

Figure 5: Phylogenetic tree of the species *Bifidobacterium adolescentis* computed from the concatenation of *purF, rpoC, rpoB, dnaJ1* gene sequences by the Maximum Likelihood methods and Tamura model integrated with Ecotype simulation results

3.7 MFA

Multiple Factor Analysis (MFA) was performed to provide an integrated representation of the strains of *Bifidobacterium adolescentis* and of the relationships between relative genes sequences and phenotypic variables. The first three dimensions of the MFA accounted for by 57.073% of the variance of the experimental data, representing 22.32%, 18.50% and 16.25% of the variance, respectively. Moreover, an extension to MFA, hierarchical MFA (HMFA) was performed to classify the strains in specific cluster, based on phenotypic and genetic data. Clusters definition was calculate by default parameters. The dendrogram of HMFA indicated that the strains evaluated could be separated into 8 clusters based on both genotypic and phenotypic variables. In HMFA each clusters can be described by different points of view: variables and/or categories, factorial axes and individuals.

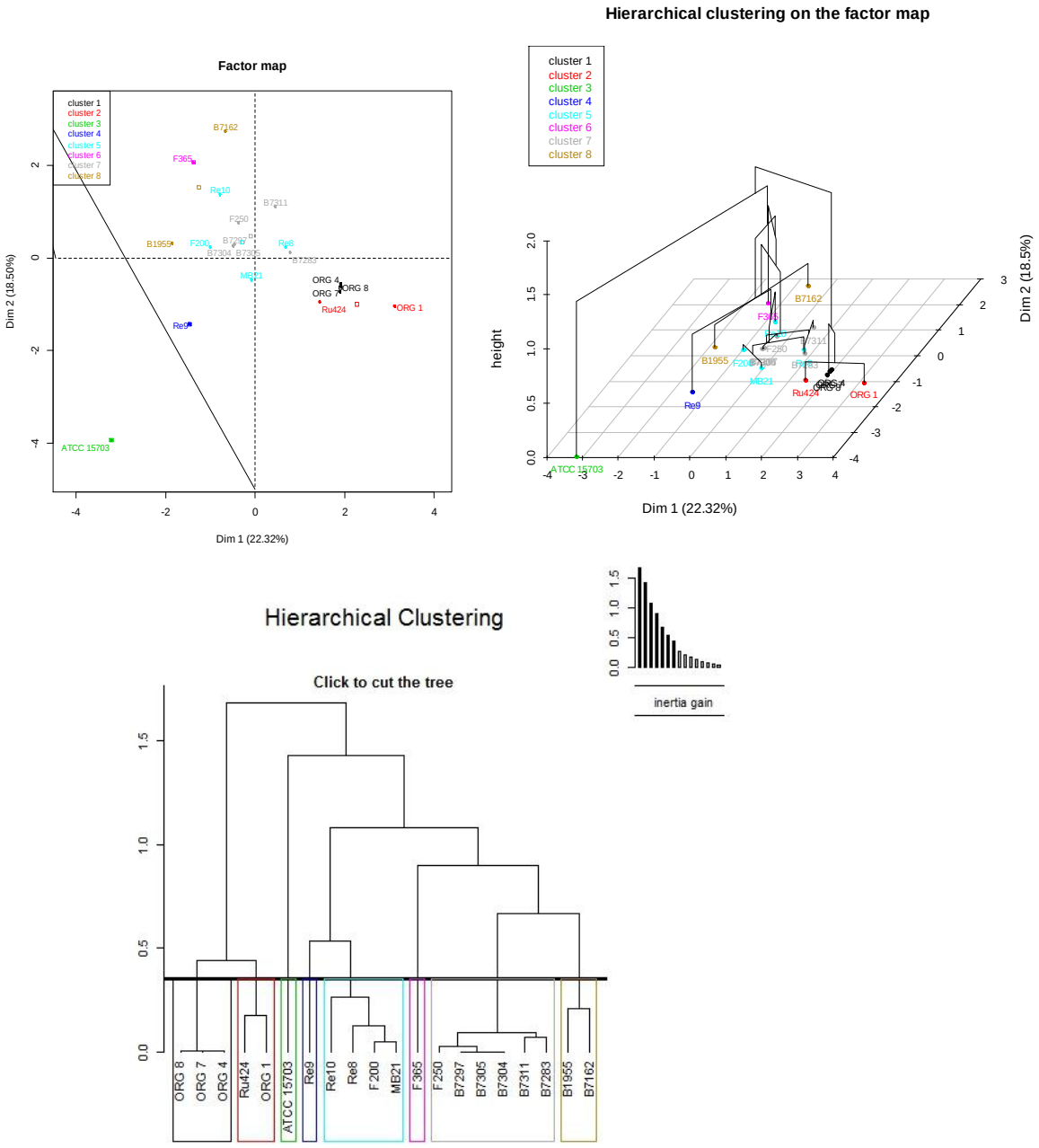

Figure 6: a) Multifactor analysis (MFA) b) and c) Hierarchical Multifactor analysis (HMFA)

In the present cluster analysis, group definition was influenced in particular by *dnaJ*, followed by *rpoC*, *rpoB* and *purF*. A first clade is composed of 3 orangutan strains and is closely related to the second group, composed of ORG1 and Ru424 (comes from Rumen of bowel). Three strains, Re09, F365 and ATCC 15703^{T} represent each one three distinct cluster. Furthermore it is remarkable that the type strains ATCC 15703^{T} is collocated (also in this cluster analysis) in a separate branch of the tree. The cluster number 5 is composed of strains of adult human origin (Re08, Re10 and MB21) and F365 a strain isolated from sewage. The group number 7 is a big clade of strains isolated from human

adult feces (B7304, B7305, B7311, B7283, B7297) and a single strain from sewage (F250). The last cluster comprised B1955 (a strain isolated from infant feces) and a strain from adult human feces (B7162); these two strains are correlate with a high *dnaJ* and *rpoC* sequence similarity.

4. Discussion

In prokaryotic kingdom, the assignment of strains to one species is generally based on measurement of genome and phenotypic similarity. Recently this kind of approach was defined as improper, considering the big level of diversity that are being uncovered in nature (Gevers et al., 2005). The concept of prokaryotic species does not represents a solid unit in terms of ecology, metabolic capabilities and gene content of their genome (Feldgarden et al., 2003; Schloter et al., 2000). A reorganization of bacterial species in smaller units has been proposed by Cohen, whose introduce the concept of ecotype as population genetically cohesive and ecologically distinct. Ecotypes are irreversibly separated from each other and represent a microbiological evolutionary unit (Cohan and Koeppel, 2008; Cohan, 2001).

B. adolescentis is one of the most abundant species of bifidobacteria in human health gut microbiota. Its related probiotic properties are of great interest and recently, researchers have highlighted an unusual intra-species diversity that characterizes this taxon. In the present study a polyphasic approach was applied on 20 *B. adolescentis* strains, in order to explore the relative intra-species variability. Firstly, all the 20 strains under investigation were identified by using molecular methods. A preliminary identification using *hsp60* PCR-RFLP (Baffoni et al., 2013) confirmed the belonging of the strains to *B. adolescentis* species and showed the presence of three distinct restriction profiles (a, b, c). The presence of multiple fingerprinting profiles into a species is absolutely uncommon, considering that usually this technique cannot discriminate at the subspecies level, as in the case of *B. longum* subsp. and *B. thermoacidophylum* subsp. (Baffoni et al., 2013). Thanks to cloning techniques, it was possible evaluate the homology levels of partial *hsp60* gene of one representing of profile b, B7304 strain. Value obtained revealed a 95% of similarity with the type strains. This *hsp60* homology value is particularly interesting because fits in the inter-subspecies range (95.5-97%) or interspecies range (80-96%) identified by Zhu et al.,(2003). Despite several unsuccessful efforts using different primers pairs and PCR setting, *hsp60* sequence could not be obtained for F365 strain, but further analyses are in progress. The presence of multiple *hsp60* PCR-RFLP profiles as well as the *hsp60* homology value provide the first indication of the wide variability of this species, suggesting a possible subdivision of this taxon in subspecies.

These preliminary identifications were further confirmed by *16S rRNA* sequence analysis. All the 20 strains were found to belong to *B. adolescentis* species, ranging from 98% to 99,72% to the *16S rRNA* sequence of type strain ATCC 15703. Ribosomal RNA (rRNA) sequence similarity is a common validated technique to rapidly identify prokaryotes, however the use of 16S rRNA as single molecular marker does not represents a useful taxonomic tools to explore bacterial evolution. Indeed this molecular marker does not consider simple stochastic variation, the effect of recombination or the horizontal gene flux (Gevers et al., 2005). In contrast a multilocus sequencing approach offered higher molecular resolution than 16S rRNA for prediction of the genetic and ecological diversity of a population (Gadagkar et al., 2005), and was shown to increase the robustness and efficacy of bacterial phylogenetic investigations (Turroni et al., 2009). For this reason a multilocus sequences analysis was performed, utilizing a set of genes composed by *dnaJ, purF, rpoC, rpoB* (Kim et al., 2010; Ventura et al., 2006). We noted the importance in using more than one gene in phylogenetic reconstruction, the discriminatory power of the concatenated tree was much more significant than that observed with each single gene. A robust phylogenetic tree was built basis on relative genes sequences concatenation, showing that 73.7% of the nodes were supported by bootstrap values greater than 80%. The same subset of sequences concatenation was also processed using *Ecotype Simulation* (ES), a program developed by Koeppel and Perry (2008). Bacterial sequences-based phylogeny is a complex study and presence of many levels of cluster and sub-cluster complicates the individuation of which level of sequence cluster should correspond to ecotypes (Cohan, 2009). For decades, demarcation of species have been performed using a universal criterion of genome content similarity, as quantified by DNA–DNA hybridization, and recently under the guidance of divergence at the 16S rRNA locus with a 1% cut-off (Stackebrant, 2011); this cut-offs have not a theoretical rationale to correspond to biologically significant clades with species-like properties, and not is it clear that any particular cut-off should apply to all bacteria (Cohan and Perry, 2007). Precisely in order to supply at these gaps *Ecotype Simulation* (ES), was applied to analyze 20 strains of *B. adolescentis* in exam. The algorithm is able also to estimate, in addition to the number of ecotypes, the rates of periodic selection and drift, the net rate of ecotype formation, so as to yield a clade's sequence diversity pattern with maximum likelihood. ES estimated a total of 7 putative ecotypes in *Bifidobacterium adolescentis* clade and suggested that ecotype formation events in this taxon are relatively rare and that putative ecotypes are regularly constrained by periodic selection. ES results are in agreement with the Multilocus approach results.

Also Rep-PCR fingerprinting techniques represents a rapid and reproducible tool for bifidobacterial differentiation, and allow to distinguish between genetically unrelated isolated and closely related bacterial strains. BOXA1R primer was recognized as suitable to discriminate Bifidobacteria at the subspecies levels (Masco et al., 2003) and for this reason it was used in this analysis. Data matrixes

obtained from BOX-profiles detection with Image Lab software were used as input in the complete-linkage clustering algorithm (Ishii et al., 2009), based on R language (R Development Core Team, 2005). In this cluster analysis *Bifidobacterium adolescentis* strains were grouped, defining 4 stable clusters (p=0.01). This methods allowed a further rapid pre-grouping screening.

Moreover each strain was tested for its ability to ferment mannitol and sorbitol, with the purpose to identify the belonging to the relative Reuter's biovars. This analysis revealed the presence of two big fermentation groups, with exclusion of two strains. On 20 strains analyzed, 10 strains were identified as biovars b (M+/S-), others 11 as c (M-/S+), Re09 as biovars d (M-/S-) and ATCC 15703^T as biovars a (M +/S+).

The tested grouping methods (Rep-PCR fingerprinting, Multilocus approach, Ecotype simulation) pointed out the presence of several clusters and in some case the results were in agreement. A Multiple Factor Analysis (MFA) was performed to provide an integrated representation of all the variables considerate in the present study. Moreover, an extension to MFA, hierarchical MFA (HMFA) was applied to classify the strains in specific cluster. HMFA identified 8 defined clusters. Despite the origin of the strains was not included in the HMFA, strains isolated from the same source clustered mainly together. This consideration remains valid for strains belonging to orangutan feces and adult human feces. On the other hand, strains isolated from sewage are divided into different clusters. It is possible to suppose that sewages does not represent a cohesive habitat for bifidobacteria strains, moreover sewage microbiota composition depends on the relative contamination source.

Unfortunately the number of strains available for each habitat considered were not homogenous (due to a lack of deposited strains isolated from different sources), and did not allow us to correctly evaluate a possible correlation between ecological origin and bacterial evolution. However an analysis of *Bifidobacterium adolescentis* phylogeny, based on all the approaches previously described, seemed to indicate a link between source and diversity.

The results of this study showed that *Bifidobacterium adolescentis* species is characterized by a wide phenotypic and genetic variability, reflected in all the variables considered in this work, and supported the idea that this species could require a new taxonomic re-organization. Moreover the type strain of *B. adolescentis,* ATCC 15703^T is defined by nonconforming characteristics, and in all the cluster analysis here performed, place itself at a significant distance to any other strains analyzed. This data is in accordance to Duranti (2013) and highlighted the controversial rules of the type strains in realistic species representation. Therefore a broad number of strains would be suitable to correctly describe the real common characteristics and properties of a species and the BUSCoB collection represents an important resource for the bifidobacteria biodiversity evaluation.

5. Conclusion

The increasing interest toward the probiotic properties of strains of *B. adolescentis*, required a correct characterization and identification of this species. In the present work several approaches have been applied to obtain an evaluation of the intrinsic variability within *B. adolescentis* species. Investigations included a multilocus approach, the use of algorithm for ecotype demarcation and different fingerprinting methods. The research showed that this species is characterized by a wide variability, highlighting the need for a new re-classification of this group and supporting the idea that a possible new taxonomic re-organization would be required. Moreover the controversial rule of the type strains in realistic species representation was clearly pointed out, since ATCC 15703^T is defined by nonconforming characteristics.

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Additional File 1: List of accession number

Strain	16S rRNA	dnaJ	purF	rpoC	rpoB
ATCC 15704	KF809871	KF840391	KF921041	KF926607	KF926625
ATCC 15705	KF809872	KF921023	KF921042	KF926608	KF926626
ATCC 15706	KF468649	KF921024	KF921043	KF926609	KF926627
MB21	KF809873	KF921025	KF921044	KF926610	KF926628
B7304	KF809874	KF921026	KF921045	KF926611	KF926629
B7311	KF809875	KF921027	KF921046	KF926612	KF926630
B7162	KF809876	KF921028	KF921047	KF926613	KF926631
B7283	KF809877	KF921029	KF921048	KF926614	KF926632
B7297	KF809878	KF921030	KF921049	KF926615	KF926633
B7305	KF809879	KF921031	KF921050	KF926616	KF926634
B1955	KF809880	KF921032	KF921051	KF926617	KF926635
F365	KF809881	KF921033	KF921052	KF926618	KF926636
F200	KF809882	KF921034	KF921053	KF926619	KF926637
F250	KF809883	KF921035	KF921054	KF926620	KF926638
ORG 1	KF468643	KF921039	KF921055	KF926621	KF926639
ORG 4	KF468644	KF921036	KF921056	KF926622	KF926640
ORG 7	KF468647	KF921037	KF921057	KF926623	KF926641
ORG 8	KF468648	KF921038	KF921058	KF926624	KF926642
Ru424	KF809884	KF921040	KF921059	KF926625	KF926643

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Accepted on Anaerobe

10.1016/j.anaerobe.2014.03.004


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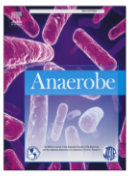

Validation of candidate reference genes in *Bifidobacterium adolescentis* for gene expression normalization

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Abstract

Normalization is an essential prerequisite for producing accurate real-time PCR expression analyses. The objective of this study is the selection of a set of optimal reference genes in *Bifidobacterium adolescentis* gene expression studies under bile exposure. *Bifidobacterium adolescentis* is a particularly abundant species in the human adults gut microbiota, exerting relevant probiotic activities. In the gastrointestinal tract, bile represents a hard challenge for bacterial survival, because of its toxic effect. The natural exposure to bile in the colonic environment induces cells adaptation and tolerance mechanisms in bifidobacteria, which determines changes in gene expression profile, influencing the expression levels of housekeeping genes. In this context, the stability of 9 putative reference genes (*cysS, purB, recA, rpoB-L, GADPH-R, 16S rRNA, glnA1, gyrA2*, *sdhA*) was examined in *B. adolescentis* exposed to bile extract, using two different software (BestKeeper and NormFinder). Both algorithms identified *gyrA2* and *sdhA* as the most stable genes under our experimental conditions, while *16S rRNA* is the least reliable HKGs. To our best knowledge, this is the first attempt to validate reference genes in *Bifidobacterium* spp. and the results offer an appropriate set of reference genes suitable for qRT-PCR studies on *Bifidobacterium adolescentis* strains under bile stress.

1. Introduction

Quantitative real-time PCR (qRT-PCR) is an effective technique for gene expression studies, which is used in molecular diagnostic, life sciences and medicine. This method is powerful and accurate, although different parameters require a particular attention. The MIQE guidelines (Minimum Information for publication of Quantitative Real-Time PCR Experiments) have been published in 2009

in order to even out qPCR experiments. The guidelines pointed out that it is essential to take care of normalization expression data to obtain reliable qPCR assays [1].

A common method for normalization is the use of a single reference gene as internal control. Generally, a reference gene is selected among housekeeping genes (HKGs), assuming that those genes are supposed to be ubiquitous, essential and constitutively expressed. However, different studies have highlighted that HKGs expression can vary considerably according to different experimental settings [2,3]. The stability of the putative reference genes should be, for this reason, verified under selected experimental conditions [2]. Moreover, Vandesompele et al. (2002) pointed out how the geometric mean of multiple reference genes is imperative to increase qPCR accuracy, while the use of a single gene could lead to relatively large errors. In the last years, many statistical algorithms have been specifically developed for reference genes evaluation (geNorm, BestKeeper, NormFinder) and are largely used to ensure reliable qRT-PCR results [4–6].

Bifidobacterium is one of the most relevant genera of the animal and human gut microbiota, which has been shown to exert beneficial effects on their physiology and pathology [7]. For this reason they are largely used as probiotics in food and pharmaceutical industry [8]. In particular, *Bifidobacterium adolescentis* represents one of the most abundant species of bifidobacteria in the gut microbiota of healthy humans [9]. The increasing interest for this species concerns the large probiotic potential of some strains; for example, recent studies have highlighted antiviral properties [10], anti-inflammatory activity [11] and folate production [12].

In the gastrointestinal tract, bacteria have to face several stress conditions, one of the most important is bile exposure that represents a hard challenge for bacterial survival. Bile is a biological detergent, synthesized in the liver and secreted into the duodenum. Although bile is reabsorbed into the terminal ileum, traces ($0.001\ g\ mL^{-1}$) remain into the food bolus and periodically contact the microbiota [13]. Because of its bactericidal effect, bile causes on microbial cells oxidative stress, protein misfolding and membrane damage [14]. How enteric bacteria are able to counteract this stress is not completely understood; certainly, deep metabolic changes are involved in this process.

Bile protection mechanisms employed by *Bifidobacteria* implicate the synthesis of bile efflux systems [14], changes in membrane protein profiles [15] and changes in carbohydrate metabolism [16], influencing the global metabolic pathways. A recent microarray study on *Bifidobacterium breve* demonstrated the strong impact of bile exposure on cell transcriptional profile, with an over expression of several genes [17]. The same effect has been observed in *B. longum* [18] where bile exposure influences the expression of an high number of genes encoding for proteins involved in stress responses, and for key components of the central and intermediary metabolism (e.g. transcription-translation machinery, gene regulation, and protein synthesis). Irrespective of the final

purpose, gene expression studies with qRT-PCR in bifidobacteria, should take into account bile influence to better simulate real gut conditions. In this context the use of appropriate tools, such as a validated reference gene, is necessary to provide reliable results.

The aim of the present work is the evaluation of the stability of some candidate reference genes in *Bifidobacterium adolescentis* ATCC 15703 exposed to bile in different phases of growth. Nine different housekeeping genes (*cysS, purB, recA, rpoB-L, GADPH-R, 16S, glnA1, gyrA2, sdhA*) were taken into account and their stability was evaluated using two independent statistical algorithms (NormFinder and BestKeeper). To date, this is the first work focusing on the validation of reference genes for transcriptional analysis in *Bifidobacterium* spp.

2. Materials and methods

2.1 Bacterial strain and culture conditions

The type strain of *Bifidobacterium adolescentis* (ATCC 15703) was obtained from ATCC Collection. Cells were grown under anaerobic conditions at 37°C in MRS (De MAN et al., 1960) broth (BioLife, Milan, Italy) supplemented with 0.05 % (w/v) L-cysteine hydrochloride (Sigma-Aldrich, Buchs, Switzerland) (MRSc). Overnight cultures of *B. adolescentis* were diluted with MRSc to reach an optical density OD_{600} of 0.5 and then treated with porcine bile extract (Sigma-Aldrich, Buchs, Switzerland) (final concentration 0.1%). Untreated cultures were used as control. Samples were incubated at 37°C, and aliquots were collected for the analyses (cell viability and RNA isolation) after 0, 30 and 60 minutes.

2.2 Evaluation of bile stress on *B. adolescentis* viability

Cell viability was evaluated with total microbial count both in bile-treated and untreated samples. 1 ml aliquots of the bacterial suspensions were removed at 0, 30 and 60 minutes, diluted serially with phosphate-buffered saline (PBS) and plated onto TPY agar plates [20]. Plates were incubated anaerobically at 37°C and the number of colony-forming units (CFUs) was determined after 48h. Three independent experiments were performed. Statistical significance ($p<0.05$) was assessed by the Student's *t*-test and analysis of variance (ANOVA) with R software [21] .

2.3 Total RNA isolation and reverse transcription

Cultures aliquots were collected at each time point and total RNAs extraction was performed using the RNeasy Mini Kit (Qiagen, Italy), according to the manufacturer's instructions. For each time point two independents replicate were considered both for treated and untreated samples.

In order to protect RNA, residual contaminant DNA was removed by using 20 U RNase-free DNAse (Qiagen, Italy). RNA elution was done in 30 µl of RNase-free water; concentration and quality were

assessed using Infinite®200 PRO Nanoquant (Tecan, Switzerland). The purified RNA was stored at -120°C for further analyses. For each sample, 100 µg of total RNA was reverse transcribed into cDNA using Omniscript RT kit (Qiagen, Italy). The obtained cDNA was stored at -20°C and used in qRT-PCR reactions as a template.

2.4 Selection of reference genes, primer design and determination of PCR efficiency

The following nine reference genes were selected for stability evaluation: *cysS*, *purB*, *recA*, *rpoB-L*, *GADPH-R, 16S, glnA1 A1, gyrA2, sdhA.* Specific primers were designed using Primer3 [22], considering the following parameters: product size range (100–200 bp), primer size (18–22 bp) and primer melting temperature (T_m 57–63 °C). The specificity of the primers was tested *in silico* using BLAST analysis against the NCBI database and through qualitative PCR on cDNA. Amplifications were performed in 20 µl volumes with 200 nM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 10 µl 2X HotStarTaq Plus Master Mix (Qiagen, Italy) and 150 ng/µl cDNA. The PCR cycle consisted of an initial denaturation of 5 min at 95°C followed by 35 cycles of denaturation (30s at 94°C), annealing (30s at 61°C) and extension (45s at 72°C), and was completed with a final elongation of 10 min at 72°C. Reactions were performed with a PCR Verity 96-well thermal cycler (Applied Biosystems, Milan, Italy) and PCR products analyzed on a 1.5% agarose gel. The amplification efficiencies were estimated with standard curves using serial dilutions of the template cDNA and calculated using the slope of a linear regression model, according to the equation: $E=10^{(-1/slope)}$ [23].

2.5 Real Time PCR

Real time PCR amplifications were processed in 48-well plate using the StepOne thermal cycler (Applied Biosystem) with SybrGreen chemistry. All reactions were performed in 20 µl volumes, containing 2 µl of 5 fold-diluted cDNA template, 250 nM of each primer and 1x Fast SYBR® Green Master Mix. All reactions were carried out in triplicate for each cDNA sample. A negative control was included in each run for each gene. The thermal profile of the reaction was 95°C for 40 sec, followed by 40 cycles of 95°C for 10 sec, and 60°C for 30 sec. A final dissociation curve was generated to determine the specificity of the reactions. The Ct values were automatically determined with default parameters.

2.6 Reference gene validation

The raw qRT-PCR amplification data were exported to Microsoft Excel. Ct-values were used for stability comparison of candidate reference genes and stability values were examined using two different algorithms: BestKeeper and Normfinder.

BestKeeper is an Excel based tool, able to compare expression levels of up to ten housekeeping genes. The algorithm is based on a pair wise correlation analysis and produce an index, the BestKeeper Index (BI), which represents the geometric mean of the Ct values of all candidate reference genes grouped together. Each gene is than compared with the BI, and that one with the highest coefficient of correlation with BI holds the highest stability [24]. NormFinder uses an ANOVA-based model to calculate a stability value for each candidate reference gene. This value is calculated on the variations of intra-inter group gene expression [25] and represents a measure of the systematic error introduced by each of the reference gene when used to normalize the data.

3. Results and discussion

The validation of reference genes is a crucial point in the normalization process in real time PCR expression analyses, considering that HKGs expression can vary considerably according to experimental settings. In the present study, nine putative reference genes were evaluated in *Bifidobacterium adolescentis* under bile exposure which represents a critical stress factor influencing metabolic pathways and therefore gene stability.

The type strain ATCC 15703 was chosen as protagonist for this study, since the availability of its full genome sequence allows the construction of suitable primers. In order to get closer to the ecological niche of bifidobacteria, the human colon, cultures of ATCC 15703 were treated with 0.1% w/v of porcine bile, due to difficulty in obtaining human bile. Porcine bile is considered an acceptable substitute because the salt/cholesterol, phospholipids/cholesterol and glycine/taurine ratios resemble the composition of human bile. The selected concentration of porcine bile is likely to approximate *in vivo* levels in the colon [13].

3.1 *B. adolescentis* viability under bile stress

Results on cell survival are shown in Figure 1. Viable microbial counts revealed differences between control and bile-treated cultures. 30 minutes after bile inoculation, viability of treated cells showed a significant decrease ($p<0.05$) compared to untreated bacteria. The difference increased after one hour incubation ($p<0.05$). Despite the low concentration used, bile influences cell survival confirming its bactericidal effect. Surviving cells, as also reported by Ruiz et al. (2012)[26] and Sanchez et al. (2005)[18], respond with a metabolic reorganization in order to minimize bile-induced damages, thus modifying their gene expression profile. In this context an evaluation of the stability of reference genes is even more essential [27].

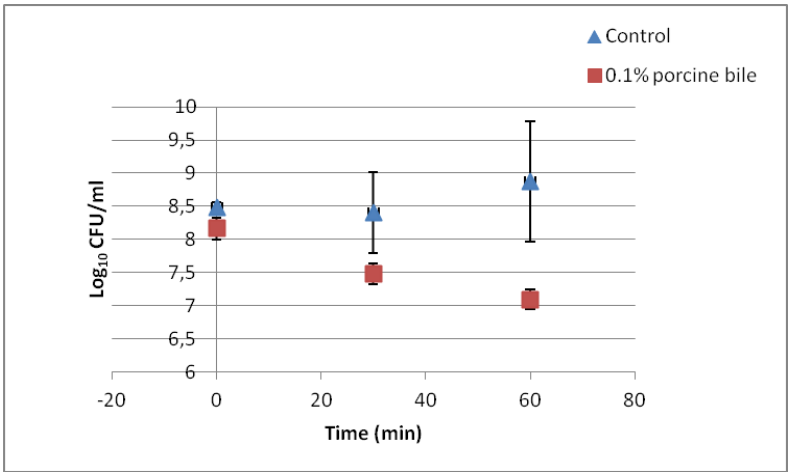

Fig 1: Trend of bacterial survival after bile salts exposure (values are expressed in log_{10} (CFU/ml) ± SD).

3.2 Selection of putative reference genes and primer design

Nine housekeeping genes were selected from commonly used reference genes to validate a qRT-PCR assay in *Bifidobacterium adolescentis.* Genes belonging to distinct biological pathways were chosen in order to avoid possible co-regulation of expression. Their full names, functions and accession numbers are listed in Table 1. All primers (except for 16S rRNA) were designed using Primer3 software [22] and were tested using BLAST analysis.

Table 1: Selected candidate reference genes, primer and PCR reactions efficiency

Gene name	GenBank accession number	Functional annotation	Primer sequences (forward/reverse)	Tm (°C)	Amplicon length (bp)	Amplification efficiency (%)	Average Cp of cDNA	References
cysS	BAF38993.1	CYSTEINYL-TRNA SYNTHETASE	5′-GGTCAGGTCGGTATCTACGC-3′ 5′-GCCTTGTCGAGGATCTTGTC-3′	84.99	173	104.39	17.81	This study
purB	BAF39335.1	ADENYLOSUCCINATE LYASE	5′-CAAGGTCAATCCGATTCGTT-3′ 5′-CTGGATGACAACATCGTTCG-3′	83.85	226	90.80	18.50	This study
recA	BAF39805.1	RECA (RECOMBINASE A)	5′-ATCTTACGCAACGCCTGACT-3′ 5′-ACCGATTCGTTGATCGTCTC-3′	83.19	203	81.17	21.95	This study
rpoB-L	BAF40100.1	DNA-DIRECTED RNA POLYMERASE BETA CHAIN	5′-GGGTGATCTTACGGTGCTGT-3′ 5′-GCCATCTTCGGTGAGAAGAG-3′	84.54	187	83.99	17.91	This study
GADPH-R	BAF39860.1	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE C	5′-GGCCTACCTGCTGAAGTACG-3′ 5′-CTCAGCGGAGGTGTAGAAGC-3′	82.91	211	97.28	21.10	This study
16 S rRNA	BAF40020.1	16S RIBOSOMAL RNA	5′-TCGCGTCYGGTGTGAAAG-3′ 5′-CCACATCCAGCRTCC**AC**-3′	82.45	243	94.50	15.27	Rinttilä et al 2004 [28]
glnA1	BAF39724.1	GLUTAMINE SYNTHETASI	5′-AGTTCAGAGACGGGTTGGTG-3′ 5′-CAAGTACGTCGTCCACGAGA-3′	84.84	242	90.75	18.41	This study
gyrA2	BAF39788.1	DNA GYRASE SUBUNIT A	5′-ATTTACGAGGCGATGGTACG-3′ 5′-GCGTGAAATCGACGGTATTT-3′	83.2	184	94.05	18.48	This study
sdhA	BAF39374.1	SUCCINATE DEHYDROGENAS FLAVOPROTEIN SUBUNIT	5′-CGGATCATATGCATGAGGTG-3′ 5′-CATCGACCCAACGGTAGACT-3′	82.61	162	84.78	18.52	This study

3.3 Amplification efficiency

RNA quality and concentration were spectrophotometrically determined (Infinite®200 PRO Nanoquant).

Yield of RNA ranged approximately 20-30 µg/µl, and A_{260}/A_{280} ratio from 1.85 to 1.97. The linear correlation coefficient (R^2) of all genes ranged 0.986–0.999. Amplification efficiencies, based on the slopes of the standard curves, ranged from 81% to 104% (Table 1). The agarose gel electrophoresis (Fig. 2a) and melting curve analysis (Fig. 2b) revealed that all primer pairs amplified a single PCR product with the expected size.

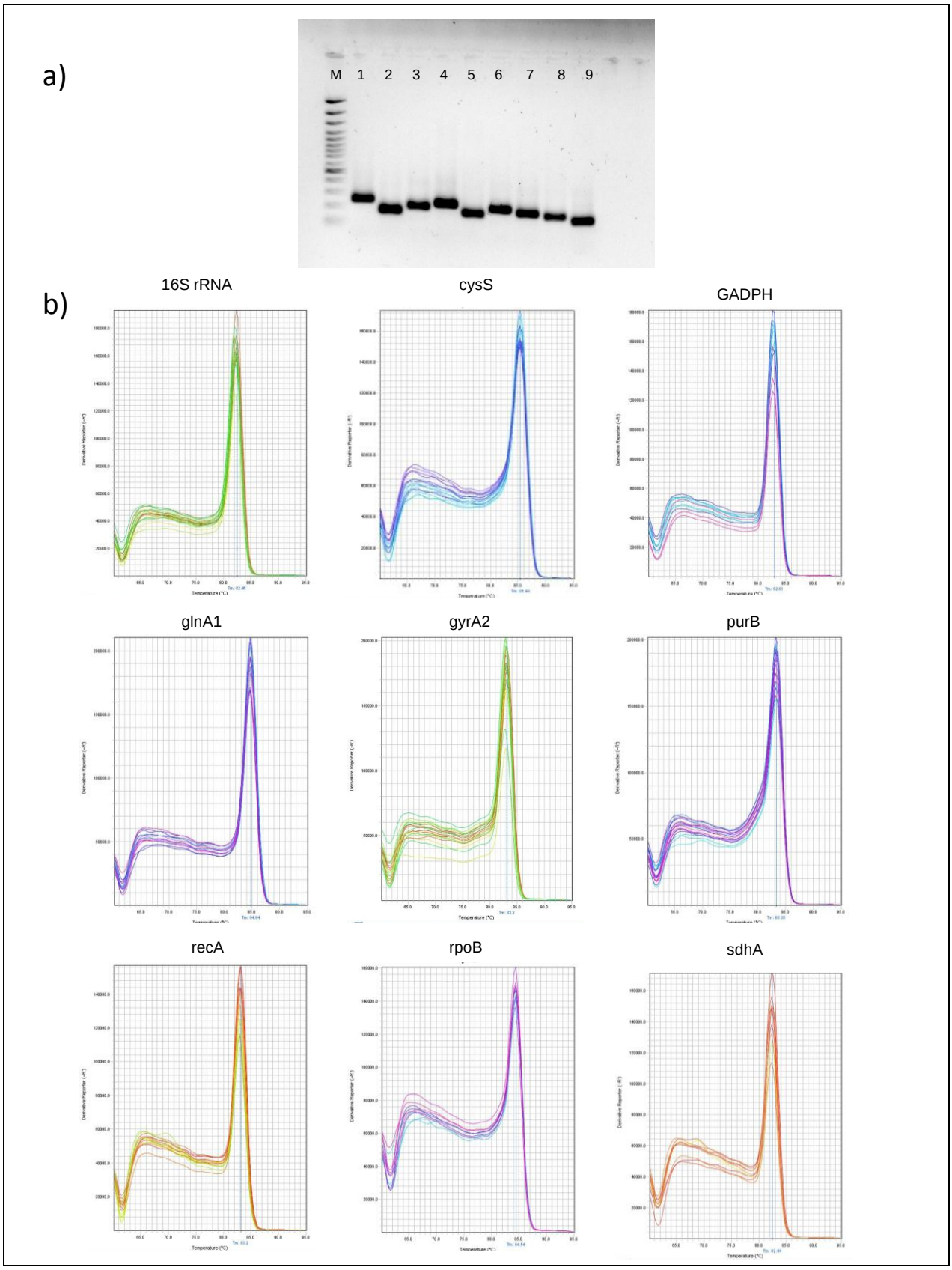

Fig 2 : Confirmation of amplicons size and primer specificity of studied reference genes

a) Agarose gel electrophoresis showing specific PCR product of the expected size for each gene (M: DNA marker; Lane1: *16S rRNA*; Lane2: *cysS*; Lane3: *GADPH*; Lane4: *glnA1*; Lane5: *gyrA2*; Lane6: *purB*; Lane7: *recA*; Lane8: *rpoB*; Lane9: *sdhA*). b) Melting curve analysis for all amplicons.

3.4 Expression stability of candidate reference genes

In order to identify the most stable reference genes among the nine selected candidates, their stability was examined and compared using two free Excel spreadsheet tools: BestKeeper and NormFinder.

BestKeeper software analysis requires genes not affected by co-regulation. Instead, NormFinder is less dependent by co-regulation of genes but could be sensitive to sampling error. The use of both programs in the study provided a robust and accurate analysis of the expression stability of the putative references genes. Extracted RNA from bile-treated cultures and controls were retrotranscribed to cDNA and qRT-PCR performed for each primer pair. The raw qRT-PCR amplification data were exported to Microsoft Excel and the averages of the Ct-values for each duplicate were calculated. Ct values were directly used in BestKeeper analysis, or transformed into relative quantification data for NormFinder analysis, according to software instructions [25]. BestKeeper analysis determines the most stably expressed genes based on the correlation coefficient to the BestKeeper Index (BI), which represents the geometric mean of Ct values of candidate reference genes. The mean Ct value obtained, GM [Ct], ranged from 15.19 to 21.57 and the highest expression value was observed for *16S rRNA*; the lowest for *recA*. Standard deviation (SD) values underlined the high variability *16S rRNA* gene, indicating the low expression stability of the gene. Conversely low SD values (concerning for examples *sdhA* and *gyrA2*) suggested their high expression stability (Table 2). In the first evaluation, the algorithm normally keeps out genes with a standard deviation higher than 1. In the present experimental conditions, the expression of *16S rRNA* gene presented a standard deviation of 1.36 (Table 2) and for this reason was not considered for the BI calculation. Its large variability would have flattened the statistical analysis of all the other genes thus, only the highly correlated control genes were then combined into the BI.

Table 2: Results from *BestKeeper* descriptive statistical analysis.

	glnA1	recA	rpoB-L	cysS	sdhA	gyrA2	purB	GADPH-R	16SrRNA
n	30,00	30,00	30,00	30,00	30,00	30,00	30,00	30,00	30,00
GM [CP]	18,10	21,57	18,08	17,58	18,31	18,24	18,34	20,96	15,19
AM [CP]	18,12	21,59	18,11	17,60	18,33	18,26	18,36	20,98	15,28
min [CP]	16,65	20,33	16,57	15,70	17,20	17,16	16,82	19,66	12,73
max [CP]	20,61	23,99	20,45	19,75	20,35	20,65	20,46	23,20	17,61
SD [± CP]	0,70	0,67	0,91	0,65	0,51	0,52	0,53	0,60	1,36
CV [% CP]	3,86	3,12	5,05	3,70	2,78	2,86	2,89	2,88	8,87

Abbreviations: N: number of samples; GM[Ct]: geometric Mean of Ct; AM[Ct]: arithmetic mean of Ct; Min[Ct] and Max[Ct]:extreme values of Ct; SD[Ct]:standard deviation of the Ct; CV[%Ct]:coefficient of variance as a percentage on the Ct level.

The eight remaining HKGs correlated well with one another and also with the BI. The best correlation between the reference genes and the BI was obtained for *gyrA2* (r=0.944) and *sdhA* (r=0.940), followed by *purB* (r=0.92) (Tab. 3). On the other hand, *rpoB-L* exhibited the worst correlation (r=0.512), followed by *cysS* (r=0.845) and for this reason they were evaluated as the least reliable HKGs after *16S rRNA*.

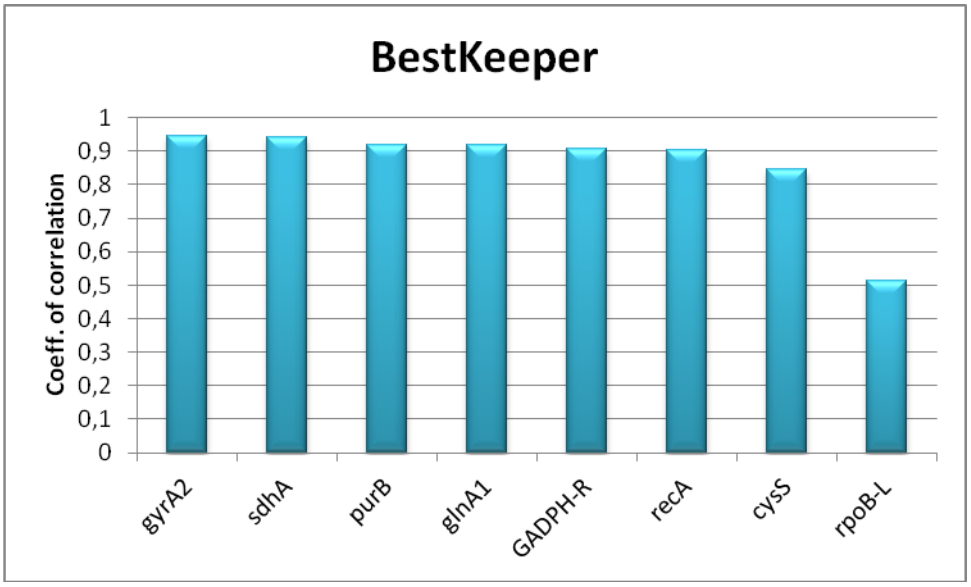

Fig3: BestKeeper analysis of the candidate reference genes. Genes are presented in decreasing order of stability from left to right

Table 3: Results from *BestKeeper* correlation analysis

BestKeeper vs	gyrA2	sdhA	purB	glnA1	GADPH-R	recA	cysS	rpoB-L
coeff. of corr. [r]	0,944*	0,94*	0,92*	0,917	0,907	0,904	0,845	0,512
p-value	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,003863

Measures of the correlation coefficients between each control gene and the BestKeeper index (16S rRNA was excluded from BI calculation due to high standard deviation)*HKGs strictly correlated between them and the index (r ≥0.92)

Additionally to BestKeeper analysis, the NormFinder program was used to grade HKGs stability. This algorithm automatically calculates a stability value for all tested genes as reported in Andersen et al. (2004). In this case, the stability is inversely correlated with the stability value (Tab. 4 Fig. 4). NormFinder ranked *gyrA2* and *sdhA* as the most stable genes, followed by *recA, GADPH-R, purB* and *glnA1*. Differently, *cysS, rpoB* and *16S rRNA* were considered as the least stable genes. It is also remarkable how *rpoB* and *16S rRNA* were again classified as the worst control genes.

Table 4: Candidate reference genes for normalization of qRT-PCR listed according to their expression stability calculated by NormFinder

Ranking Order	Gene Name	Stability Value
1	gyrA2	0,023
2	sdhA	0,035
3	recA	0,040
4	GADPH-R	0,044
5	purB	0,047
6	glnA1	0,049
7	cysS	0,139
8	rpoB	0,226
9	16S rRNA	0,350

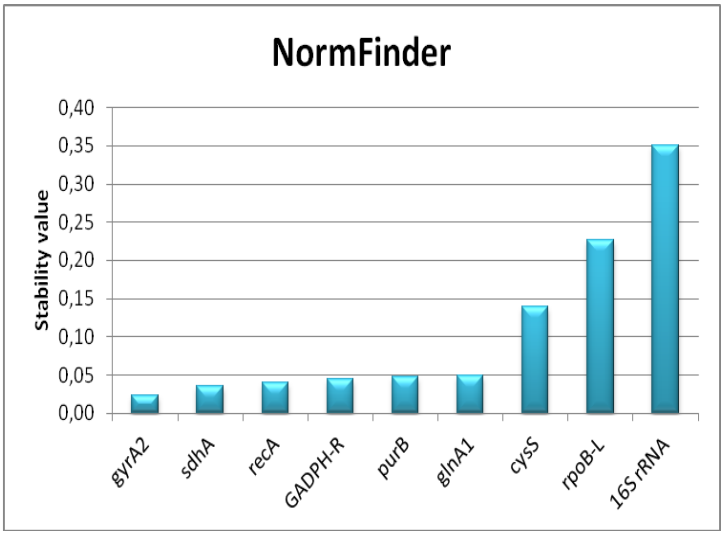

Fig 4: NormFinder analysis of the candidate reference genes. Genes are presented in an increasing order of stability from left to right.

Overall, the two software, based on different algorithms and analytical procedures, produced highly comparable results, although there are little differences in the ranking order, both of the programs agree on the same two most stable and the two most unstable genes.

4. Conclusions

The validation of reference genes represents a crucial point in the normalization process. To our best knowledge, this study is the first attempt to validate a set of reference genes in *Bifidobacterium* spp. for qRT-PCR analyses. In particular, this work aimed to evaluate nine putative reference genes, belonging to distinct biological pathways, in *Bifidobacterium adolescentis* under bile exposure, which is an important stress factor influencing gene expression profiles. The two algorithms tested (BestKeeper and NormFinder), based on different statistical approach, produced comparable results.

Both of them revealed that *gyrA2* and *sdhA* possess the most stable gene expressions across the different experimental conditions, whereas *16S rDNA* and *rpoB* are the two least stable. Moreover, the extreme instability of *16S rDNA* confirms the bile-effect on the transcriptome, demonstrating how the choice of control genes in qRT-PCR analyses can affect the interpretation of bacterial gene expression levels. In conclusion, the use of *gyrA2* and *sdhA* as reference genes can be suitable for gene expression studies focusing on *Bifidobacterium adolescentis*, in order to enhance the robustness of qRT-PCR when bile stress is considered, being a critical factor in the gut environment for bacterial survival.

Considering the increasing interest towards *B. adolescentis* strains as probiotic microorganisms, further gene expression studies are advisable to have a deep insight into their metabolism, and this study could represent an useful baseline for the required normalization process.

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Paper 5

Under submission

Gene expression and folate production in *Bifidobacterium adolescentis* strains

Verena Stenico, Loredana Baffoni, Thomas Andlid, Paola Matterelli, Bruno Biavati

Abstract

Folates (vitamin B9) are essential vitamins in humans and its deficiency may contribute to the onset of several diseases. Humans and other mammals are unable to synthesize *ex novo* folate and obtain it only from exogenous sources. The gut microbiota has been recently identified as an important source of this vitamin and it is demonstrated that folate produced by gut microbiota can be absorbed in the large intestine. Bifidobacteria, an important group of intestinal microorganisms, produce vitamins of B group, including B9. The objective of this work was the investigation of folate metabolism in *Bifidobacterium adolescentis* during several growth phases, in different strains and in presence of bile, by means of a double approach. Quantitative analyses on folate content were integrated with expression studies on genes involved in folate related pathways. All *Bifidobacterium adolescentis* strains tested showed a high folate content, with a peak during the exponential growth phase. With this study we have also demonstrated that bile stress has a strong influence on bifidobacterial metabolism, significantly affecting bacterial survival, folate content, as well as relative gene expression. To our knowledge this study is the first attempt to have a deep insight into *Bifidobacterium* folate metabolism and reveals *Bifidobacterial adolescentis* as a promising specie in terms of folate content, this aspect represents an added value in the selection of this species to be used as probiotic.

Key words: folate, folic acid, *Bifidobacterial adolescentis, qRT-PCR, HPLC*

1. Introduction

Folates (vitamin B9) are water soluble vitamins and are essential cofactors for one carbon transfer reactions in cells. The common term “folates” is used to indicate all the chemical forms of folate, including the different oxidation states and the polyglutamic forms. The basic molecular structure of folate consists of a pteridine ring (DHPP) conjugated to a para-aminobenzoic acid unit (pABA), which is linked to a chain of L-glutamate residues. Different one carbon units (CH_3, CH_2, CH, CHO, HCHO) can be linked at the N_5 and N_{10} position of the pteridine ring, influencing the stability of the compounds. Folate is essential for methylation, for the synthesis of nucleic acid and some aminoacids, representing a crucial factor for cellular growth and replication (Crider et al., 2012). Folate deficiency

in humans may contribute to the onset of several diseases such as anaemia, cancer, cardiovascular disease, neurological problems as well as defects in embryonic development (Iyer and Tomar, 2009). The daily recommended intake (RDA) for folate in the European Union is 400 μg/day for adults, but this value increases until 600 μg/day in women planning a pregnancy, in order to prevent possible neural tube defects in the fetus (FAO/ WHO, 2002). Humans and other mammals are unable to synthesize *ex novo* folate and obtain it only from exogenous sources, via intestinal absorption. Folate is widely distributed in the biological world, and recently it has been demonstrated that the gut microbiota is an important source of this vitamin, and it can be absorbed in the large intestine actively contributing to the human folate intake (Asrar and O'Connor, 2005; Aufreiter et al., 2009; Strozzi and Mogna, 2008). Bifidobacteria, an important group of beneficial intestinal microorganisms, produce vitamins of B group (including vitamin B9). The capability of these microorganisms to produce folates has been explored in recent studies (D'Aimmo et al., 2012; Pompei et al., 2007a) showing large differences in vitamin content among strains. The level and the composition of folates seem therefore strain-dependent, however high levels of folate have been found in several *B. adolescentis* strains (e.g. 6415 μg per 100 g dry matter in TPY medium), with concentrations comparable to folate levels in yeast (D'Aimmo et al., 2012). In addition some *B. adolescentis* strains were able to grow in folate-free-medium (FFM), suggesting a total autonomy in folate synthesis (D'Aimmo et al., 2012). This piece of data is supported by an *in-silico* analysis of fully sequenced *B. adolescentis* ATCC 15703^T genome, that revealed the presence of the entire set of genes involved in folate biosynthesis, including those related to the folate precursor production. The distribution of folates in the extracellular environment has been described in Pompei et al. (2007). Using a microbiological assay. Pompei reported that only a small fraction of total folate production, ranged between 9-38%, is stored in the intracellular environment. This study supposed moreover that folate biosynthesis, in some *B. adolescentis* strains, is not subjected to feed back regulation in presence of exogenous source of folate, reaching a final concentration 50-fold higher than the requirement (MB239 and MB227 strains) (Pompei et al., 2007a).

At present, only few studies have been published regarding bifidobacteria and folate production, focusing mainly on folate levels in different strains, and on the impact of medium composition on folate content (D'Aimmo et al., 2012; Pompei et al., 2007a, 2007b; Strozzi and Mogna, 2008). Many aspects, as the relation between folate content and gene expression or the real production of the vitamin in the gastrointestinal tract (GI tract) are still not completely understood. Intestinal bifidobacteria are supposed to produce folate *in situ*, however in the GI tract bacteria encounter several environmental stresses, and one of the most important stress is bile exposure which causes deeply metabolic effects.

Bile is a heterogeneous mixture of organic and inorganic compounds (bile salts, phospholipids, cholesterol, bilirubin and protein) produced by the liver and secreted into the duodenum during the digestion process. Bile exerts an essential physiological role in human health, facilitating the emulsification and absorption of liposoluble nutrients, and exerts a strong antimicrobial activity, contributing to the microbiota balance (Begley et al., 2005). After the release of bile into the small intestine, bile salts are absorbed in large part by the ileum, and return to the liver by way of the portal vein, thus completing a portal enterohepatic circulation (Small, 1972). Despite this reabsorption process, traces of bile (0.001 g mL−1) remain into the food bolus and periodically contact the microbiota (Begley et al., 2005). As a consequence of their detergent–like properties, bile salts induce membrane damages, influencing the membrane permeability, and induce also microbial cells oxidative stress and protein misfolding. Bifidobacteria, in the colonic environment, are regularly exposed to bile stress conditions (because of the passage of food bolus) activating mechanisms to neutralize bile toxicity. In order to ensure cell integrity, a variety of survival strategies have been especially developed and implicate the synthesis of bile efflux systems (Gueimonde et al., 2009), the production of exocellular polymers (Ruas-Madiedo et al., 2005), changes in membrane protein profiles (Margolles et al., 2003) and in carbohydrate metabolism (Ruas-Madiedo et al., 2005); influencing the central metabolic pathways. *Bifidobacterium* metabolism is significantly affected by bile exposure, it is therefore hypothetical that folate biosynthesis and at the end the folate content could be influenced.

The purpose of this work is to gain a fundamental understanding of folate metabolism in *Bifidobacterium* spp. *Bifidobacterium adolescentis* ATCC 15703^{T} was selected as model organism, considering its folate related characteristics, and bile influence was also included in this study. In addition four *B. adolescentis* strains have been selected to evaluate possible differences in folate content. The strains were chosen considering the MFA results obtained in the previous work on *B. adolescentis* intra-species variability (Paper 3). A double approach was planned to investigate the *Bifidobacterium* folate metabolism: firstly a quantitative approach using HPLC technique, and secondly a transcriptional analysis by qRT-PCR. To the best of our knowledge this is the first attempt to study the transcriptome of *Bifidobacterium adolescentis* in relation to folate production.

2. Materials and methods

2.1 Bacterial strains

Bifidobacterium adolescentis ATCC 15703^T was obtained from ATCC Collection (American Type Culture Collection, Manassas, VA). *Bifidobacterium adolescentis* strains F365, B7311, B7162, MB21 belong to BUSCoB collection (Bologna University Scardovi Collection of Bifidobacteria, Bologna, Italy).

2.2 Culture conditions

B. adolescentis ATCC 15703^T was grown under anaerobic conditions at 37°C in MRS (De MAN et al., 1960) broth (BioLife, Milan, Italy) supplemented with 0.05 % (w/v) L-cysteine hydrochloride (Sigma-Aldrich, Buchs, Switzerland) (MRSc). 500 µl of an overnight culture of *B. adolescentis* ATCC 15703^T were inoculated in 10 ml MRSc tubes. Tubes were incubated at 37°C, and aliquots were collected for the analyses (cell count, RNA isolation, folate quantification) at different optical densities (OD_{600}) to compare folate levels in different growth phases. To compare folate production levels of different *B. adolescentis* strains (B7311, B7162, F365, MB21, ATCC 15703^T) cells were grown under anaerobic conditions at 37°C in MRSc. Cells were collected at OD_{600} 0.8 for the analyses (cell viability, RNA isolation, folate quantification).

To perform bile assay cells were grown under anaerobic conditions at 37°C in MRSc. Overnight cultures of *B. adolescentis* ATCC 15703^T were adjusted at OD_{600} 0.5 and then treated with porcine bile extract (Sigma-Aldrich, Buchs, Switzerland) (final concentration 0.1%). Untreated cultures were used as control. Samples were incubated at 37°C, and aliquots were collected for the analyses (cell viability and RNA isolation) after 0, 30 and 60 min.

2.5 *B. adolescentis* ATCC 15703^T viability under bile stress

Cell viability was evaluated with total microbial count both in bile-treated and untreated samples. 1 ml aliquots of the bacterial suspensions were removed at 0, 30 and 60 min, diluted serially with phosphate-buffered saline (PBS) and plated on TPY agar plates (Biavati et al., 1992). Plates were incubated anaerobically at 37°C and the number of colony-forming units (CFUs) was determined after 48h. Three independent experiments were performed. Statistical significance ($p<0.05$) was assessed by the Student's *t*-test and analysis of variance (ANOVA) with R software (R Development Core Team, 2005).

2.6 Folates analysis by HPLC

Intracellular and extracellular folate was determined using a validated high-performance liquid chromatography (HPLC) method (Patring et al 2005). In order to avoid folates degradation, samples

were handled by protecting from light, under nitrogen atmosphere and stored on ice. Cell extracts and culture supernatants were prepared following the methods described by D'Aimmo et al., (2012). Tetrahydrofolate sodium salt (H_4 folate), 5-HCO-5,6,7,8,-tetrahydrofolate sodium salts (HC0-H_4folate) and

5 CH3- 5,6,7,8,-tetrahydrofolate sodium salts ($5CH_3H_4$folate) were used as references for analyses. The purity of all standards was checked according to the procedure of Van Den Berg et al. (1994) using molar extinction coefficients reported by Eitenmiller et al. (2007). The concentration of all standard stock solutions was corrected for purity.

2.7 Total RNA isolation and reverse transcription

Bacterial culture aliquots were collected in duplicate for each experiment and at each treatment time point and total RNAs extraction was performed using the RNeasy Mini Kit (Qiagen, Italy), according to the manufacturer's instructions. In order to protect RNA, residual contaminant DNA was removed by using 20U RNase-free DNAse (Qiagen, Italy). RNA elution was done in 30 µl of RNase-free water; concentration and quality were assessed using Infinite®200 PRO Nanoquant (Tecan, Switzerland). Purified RNA was stored at -120°C for further analyses. 100 µg of total RNA were reverse transcribed using Omniscript RT kit (Qiagen, Italy). The obtained cDNA was stored at -20°C and used for qRT-PCR reactions.

2.8 Selection of key genes, primer design

Specific primers were designed using Primer3 (Rozen and Skaletsky, 2000), considering the following parameters: product size range (100–200 bp), primer size (18–22 bp), primer melting temperature (T_m 57–63 °C). The specificity of the primers was tested *in silico* using BLAST analysis against the NCBI database and through qualitative PCR on cDNA. Amplifications were performed in 20 µl volumes with 200 nM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 10 µl 2X HotStarTaq Plus Master Mix (Qiagen, Italy) and 150 ng/µl cDNA. The PCR cycle consisted of an initial denaturation of 5 min at 95°C, 35 cycles of denaturation (30s at 94°C) annealing (30s at 61°C) and extension (45s at 72°C), and a final elongation of 10 min at 72°C. Reactions were performed with a PCR Verity 96-well thermal cycler (Applied Biosystems, Milan, Italy). PCR products were analyzed on a 1.5% agarose gel.

PCR efficiency was estimated with standard curves using serial dilutions of the template cDNA and calculated using the slope of a linear regression model, according to the equation: $E=10^{(-1/slope)}$ (Ramakers et al., 2003).

2.9 qRT-PCR

Real time PCR amplification reactions were performed in 48-well plate using the StepOne thermal cycler (Applied Biosystem) with SybrGreen chemistry. All reactions were performed in 20 µl volumes, contained 2 µl of 5 fold-diluted cDNA template, 250 nM of each primer and 1x Fast SYBR® Green Master Mix. All reactions were carried out in triplicate for each cDNA sample. A negative control was included in each run for each gene. The validated reference gene *sdhA1* (PAPER 3), encoding for a succinate dehydrogenase flavoprotein subunit, was used as internal control. The amount of each gene transcript, normalized to the internal reference *sdhA1*, was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The thermal profile of the reaction was 95°C for 40 sec, followed by 40 cycles of 95°C for 10 sec, and 60°C for 30 sec. Amplicon specificity was verified by melting curve analysis (60 to 95 °C) after 40 cycles. Ct values were automatically determined with default parameters

3.0 Statistical analysis

Statistical significance ($p<0.05$) was assessed by the Student's *t*-test and analysis of variance (ANOVA) with R software (R Development Core Team, 2005). Tukey's test was also used to compare the folate content results between treatments at different time points after bile exposure and in different growth phases.

3. Results

The available literature concerning folates and *Bifidobacterium* spp. revealed that *B. adolescentis* is one of the most promising species in terms of folate production. For this reason the type strain of *B. adolescentis* (ATCC 15703^T) was chosen as model organism for this study. Moreover the availability of its full genome sequence allowed an *in silico* study of folate pathways and the construction of suitable primers for qRT-PCR studies.

3.1 Selection of genes and primer design

Nine key genes involved in folate biosynthesis and in the one-carbon pool pathway were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa et al., 2012). Reactions and enzymes considered in the present study are illustrated in figure 1 and table 1.

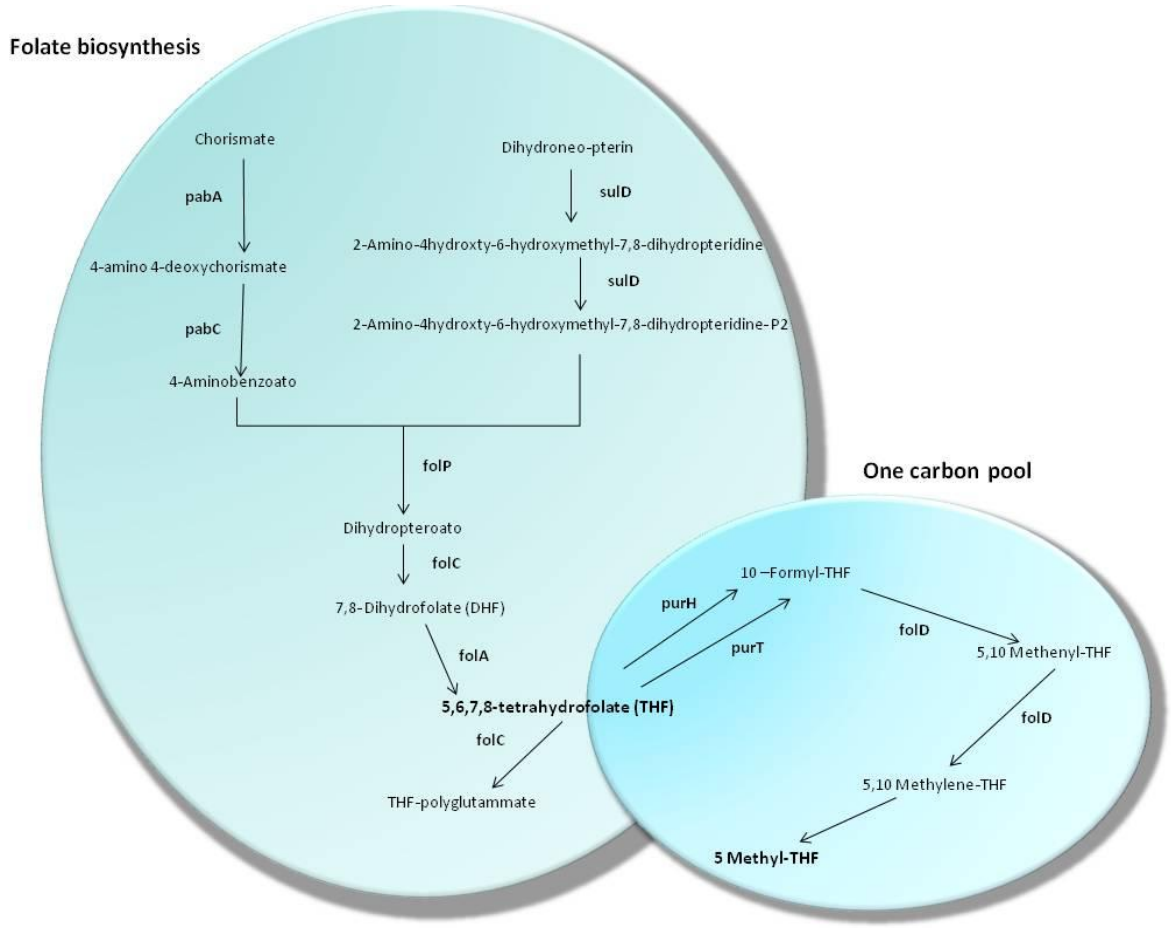

Figure 3: Reactions, enzymes and context of folate biosynthesis in *B. adolescentis*

A validated reference gene (*sdhA1*) was used as endogenous control for the normalization process (see paper n°3). *betA*, a gene encoding for a bile efflux transporter protein, has been selected as positive control in bile stress assay considering that Gueimonde et al., (2009) reported an up-regulation of this gene at sub-inhibitory concentrations of bile. In addition, *dppD* gene, encoding for an ABC transporter ATP-binding protein, was monitored. Previous studies identified in *Lactococcus lactis* a specific folate transporter protein in the proximity of *folC* (called *folT*). The analysis of *B. adolescentis* ATCC 15703 genome revealed an ABC transport protein next to *folC* gene, called *dppD*. All primers were designed using Primer3 software (Rozen et al., 2000) and were tested using BLAST analysis (Table 1).

Table 1: Selected genes and newly designed primer

Gene name	GenBank accession number	Functional Annotation	Primer sequences forward	reverse	Amplicon length (bp)	References
folA/dfrA	BAD_0389	DIHYDROFOLATE REDUCTASE	TCCCCAAAACCTTCTCTGTG	TCATGCGAGATGACGATGTT	232	This study
folC	BAD_1088	DIHYDROFOLATE SYNTHASE / FOLYLPOLYGLUTAMATE SYNTHASE	GCTTCTTCGAAGTGCTCACC	CTTGATGATGCCGACCTTTT	215	This study
folP	BAD_0411	DIHYDROPTEROATE SYNTHASE 1	CCTGTACATCGTGCAGCATT	GGGTCGATGATGACCTGTTC	177	This study
folD	BAD_0673	METHYLENE-TETRAHYDROFOLATE DEHYDROGENASE/METHYLENE-TETRAHYDROFOLATE CYCLOHYDROLASE	TGATCGATCCCAAGAAG GAC	CGCCTTACGAGTCAACAA CA	223	This study
sulD/ fol BF	BAD_0412	ALDOLASE-PYROPHOSPHOKINASE	GTATTTCGCCGCTGTACCAT	ATGTCCACAAGGTCGAGGTC	163	This study
pabA	BAD_0037	PARA-AMINOBENZOATE SYNTHETASE COMPONENT II	GCTACCATTCCCTGGCAGT	TCAACAACCTTAGGCTGCAA	244	This study
pabC	BAD_0076	4-AMINO-4-DEOXYCHORISMATE LYASE	AGTTCGATCGTTCTGGCAGT	ATTCGCAAGACGCTTCAGAT	216	This study
purH	BAD_0811	PHOSPHORIBOSYLAMINOIMIDAZOLECARBOXAMIDE FORMYLTRANSFERASE / IMP CYCLOHYDROLASE	CCTGCGTATCCTGAAGGT GT	AGCGATAAGTATGGCGTT GG	225	This study
purT	BAD_0525	PHOSPHORIBOSYLGLYCINAMIDE FORMYLTRANSFERASE	CCGGCTCTCTGGAAGAA CTA	ACGGTCAGTACGGTCAG CTC	234	This study
dppD	BAD_1091	ABC TRANSPORTER ATP-BINDING PROTEIN	CGTATTGGCCAGAAACTGGT	CCTACCAGACCGAGCACTTC	200	This study
betA	BAD_1491	EFFLUX TRANSPORTER PROTEIN	GTGACATGACGGTCTGGTTG	GCCGTTACCAACAGAAGGAA	164	This study
sdhA1	BAF39374	SUCCINATE DEHYDROGENAS FLAVOPROTEIN SUBUNIT	CGGATCATATGCATGAGGTG	CATCGACCCAACGGTAGACT	162	Stenico et al. 2014

3.2 Folate levels and growth phases

3.2.1 Folate profile in *B. adolescentis* culture

The results presented in Figure 2 concern the intracellular folate content as function of cellular growth. Samples were collected for the analyses at different OD values to evaluate the intra and extracellular folate content during the different growth phases. The dominating form of folate was $5CH_3H_4$folate at each stage of growth; low amounts of H_4folate were also detected. Concerning $5HCOH_4$folate it was not quantifiable due to insufficient sensitivity of the method.

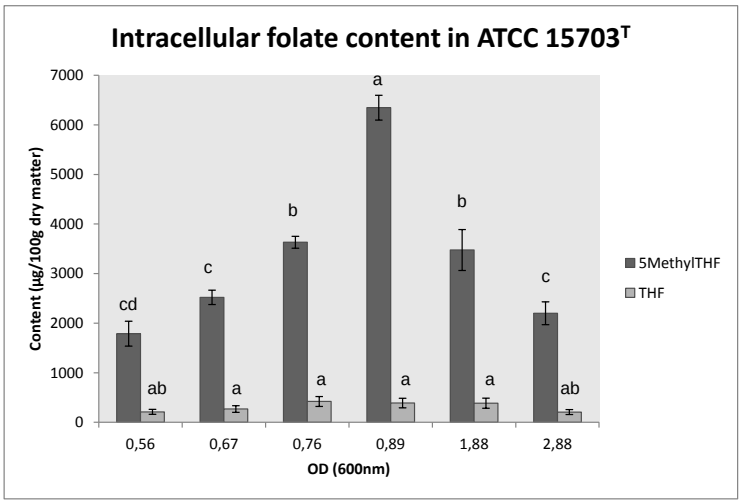

Figure 4: *B. adolescentis* ATCC 15703^T intracellular folate content in different growth phases. Different letters indicate significant differences at p<0.05

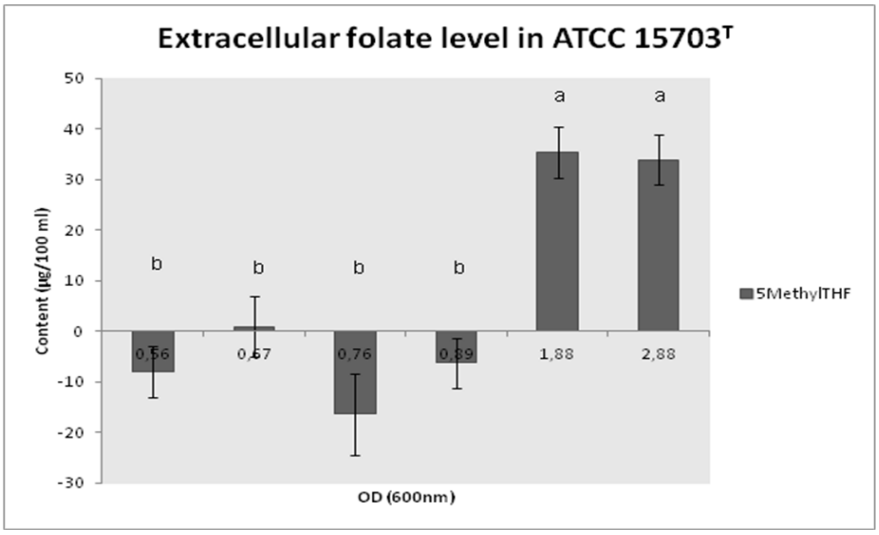

Figure 5: *B. adolescentis* ATCC 15703^T extracellular folate level in different growth phases. Different letters indicate significant differences at p<0.05

Large variations in folate content were observed at different stages of growth. Data showed that folate levels increased in the intracellular environment during the exponential phase. The highest level of folates (6736 ± 500 µg/100g dry matter) was found at OD_{600} 0.89, corresponding with the late exponential phase and the higher plate count (OD_{600} =1 * 10^9 cfu/ml) (Figure 4). This growth period is characterized by rapid cell doubling and in this situation cells require high amounts of vitamins (reference). Instead during the plateau phase and the death phase the intracellular folate content decreased (Figure 2). This is in accordance with studies in yeast, in which high cell activity is correlated to high amounts of $5CH_3H_4$folate (Hjortmo et al., 2008).

Regarding the folate level in supernatants an opposite situation could be found (Figure3). Quantities were normalized by subtracting folate levels of the medium. The medium itself, MRSc, was found to contain 53 ng*$mL^{-1}$of $5CH_3H_4$folate, no H_4folate was detected. In the exponential phase, calculated folate levels are under the MRSc folate level, this means that cells consume the vitamin present into the medium to respond to the replication stress. During the plateau phase and the death phase the extracellular level increases. The only folate form detectable in the supernatants was the $5CH_3H_4$folate.

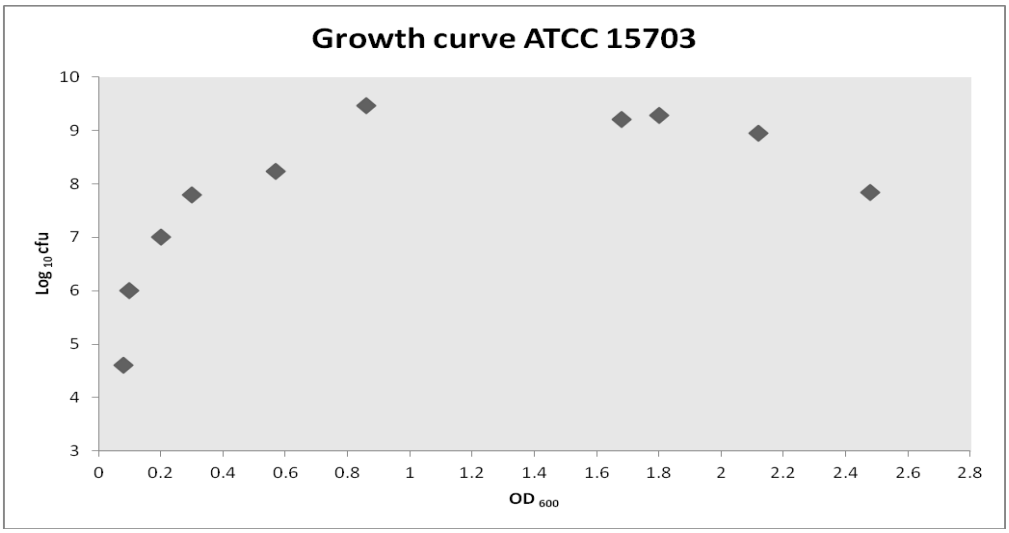

Figure 6 : Growth curve of *B. adolescentis* ATCC15703, the graph shows values of log_{10} CFU/ml for different optical densities.

3.2.2 Gene expression

The expression of the selected nine genes was assayed at different OD points and then normalized to the basal expression of *B. adolescentis* ATCC 15703 ^{T} at OD 0.3 (Figure 5).

The principal genes involved in folate production (*folA, folC* and *folP*) were constantly expressed during all growth phases analyzed, showing a constitutive expression of the folate pathway; whereas genes involved in the production of folate precursors were significantly down regulated compared to the basal expression. Indeed *sulD*, a gene encoding for aldolase-pyrophosphokinase, was significantly down regulated at OD 0.6 ($p<0.000$) and OD 0.9 ($p<0.005$) together with *pabC,* a gene involved in the biosynthesis of the para-aminobenzoic acid unit (pABA) ($p<0.005$).

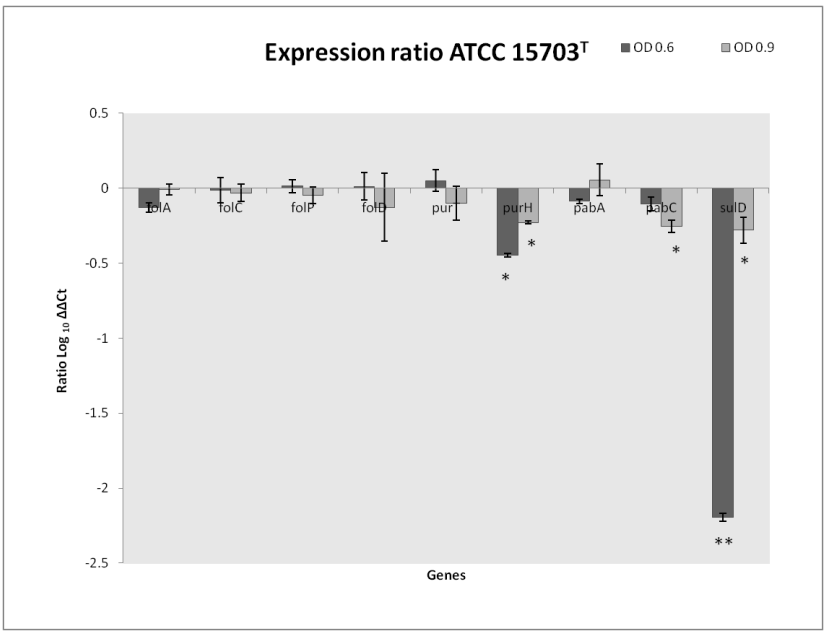

Figure 7: Relative expression levels of *B. adolescentis* genes in different growth phases. Values are normalized to the expression level obtained at OD 0.3. * and ** indicate significativity at p<0.01 and p<0.001 levels respectively.

3.3 Folate levels in different strains of *Bifidobacterium adolescentis*

3.3.1 Folate content

Five different strains of *B. adolescentis* (ATCC 15703 ^{T}, B7311, MB21, F365, B7162) were screened for their ability to produce folates. Cells were collected from overnight (12h) cultures at the same optical density (OD_{600} = 0.89), which correspond to the maximum peaks of folate content in *B. adolescentis* ATCC 15703^{T}. To the best of our knowledge, the production of folate is highly strain-dependent and the type strain of *B. adolescentis* (ATCC 15703^{T}) was found to be one of the best bifidobacterial strains for folate content (6415 µg per 100 g of dry matter) (D'Aimmo at al 2012).

The screening results (Figure 6) showed significant differences in folate content among strains ($p<0.05$), identifying three distinct groups. The total folates content ranged from 3303 to 9742 µg/100 g dry matter. $5CH_3H_4$folate was found to be the main folate form in *Bifidobacterium adolescentis* strains. Small amounts of H_4folate were recognized, but in some cases this form is completely undetectable.

Bifidobacterium adolescentis B7311 and MB21 contained the highest amounts of total folate: 9742 (±288) and 9040 (±102) µg/100g dry matter, respectively. *B. adolescentis* ATCC 15703^{T} showed an

intermediate content, 6736 (±500) µg/100g dry matter, while strains F365 and B7162 contained low levels of total folate, 3611(±508), 3303(±30) µg/100g dry matter, respectively.

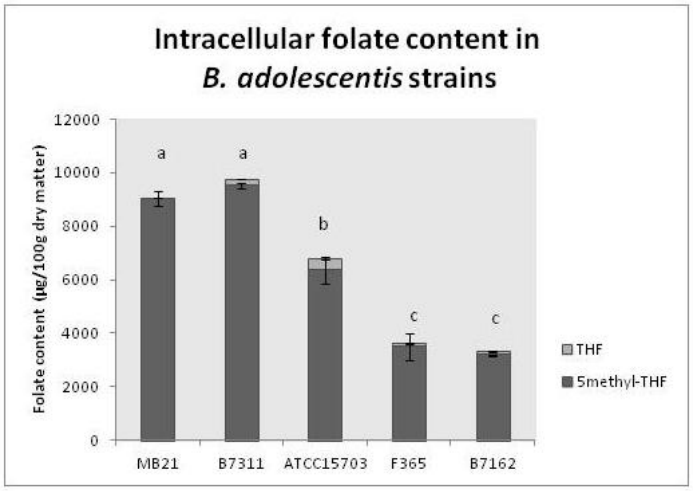

Figure 8: Intracellular folate content in *B. adolescentis* strains (evaluated at OD_{600} 0.8). Different letters show significant differences (p<0.05)

3.3.2 Gene expression analysis in different strains of *Bifidobacterium adolescentis*

Being the strain *B. adolescentis* B7311 the higher folate producer evaluated in this study, its gene expression profile was compared to the ATCC 15703^{T} profile, trying to evidence a possible relationship between high folate content and gene expression (Figure 7).

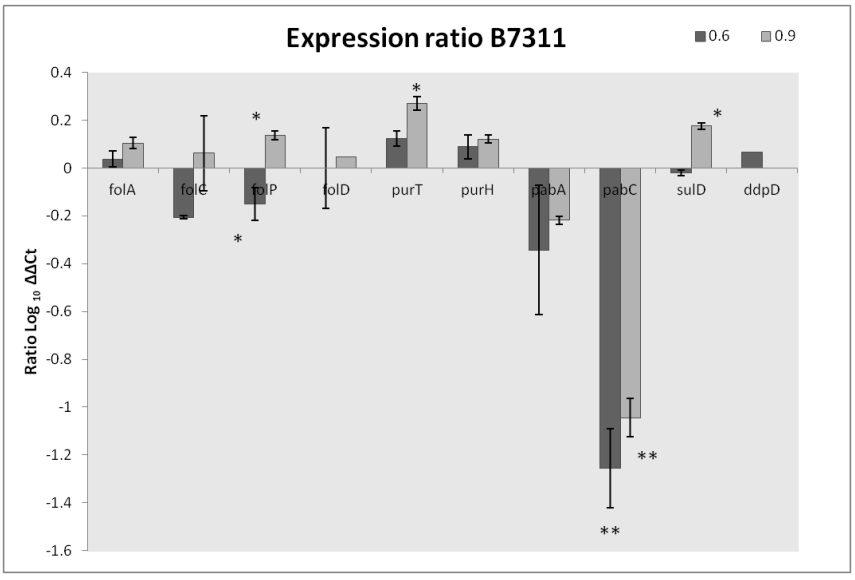

Figure 9: Relative expression levels of *B. adolescentis* B7311 genes in different growth phases. Values are normalized to the expression level obtained from *B. adolescentis* ATCC 15703 ^{T}.
*** and ** indicate significativity at p<0.01 and p<0.001 levels respectively.**

The relative expression levels of *pabC* in both growth phases (OD_{600} 0.6 and 0.9) were significantly lower ($p<0.000$) in B7311 than in ATCC 15703^T. *pabC* coding for the enzyme 4-amino-4-deoxychorismate lyase involved in the biosynthesis of the para-aminobenzoic acid unit, an important precursor for the folate production. *sulD, purT* and *folP* genes displayed slight significant differences in their expression levels ($p<0,005$). The obtained results however did not explain the folate variation observed in the two strains.

3.4 Evaluation of folate content, cell survival and gene expression under bile exposure

In order to get closer to the ecological niche of bifidobacteria, the human colon, cultures of *B. adolescentis* ATCC 15703^T were treated with 0.1% of porcine bile, due to difficulty in obtaining human bile. Porcine bile is considered an acceptable substitute because the salt/cholesterol, phospholipids/cholesterol and glycine/taurine ratios resemble the composition of human bile. The selected concentration of porcine bile (0.1% w/v) is likely to approximate *in vivo* levels in the colon (Begley et al., 2005).

3.4.1. *B. adolescentis* viability under bile stress

Results on cell survival are shown in Figure 8. Viable microbial counts revealed differences between control and bile-treated cultures. 30 minutes after bile inoculation, viability of treated cells showed a significant decrease ($p<0.05$) compared to untreated bacteria. The difference increased after one hour incubation ($p<0.05$). Despite the low concentration used, bile influenced cell survival confirming its bactericidal effect.

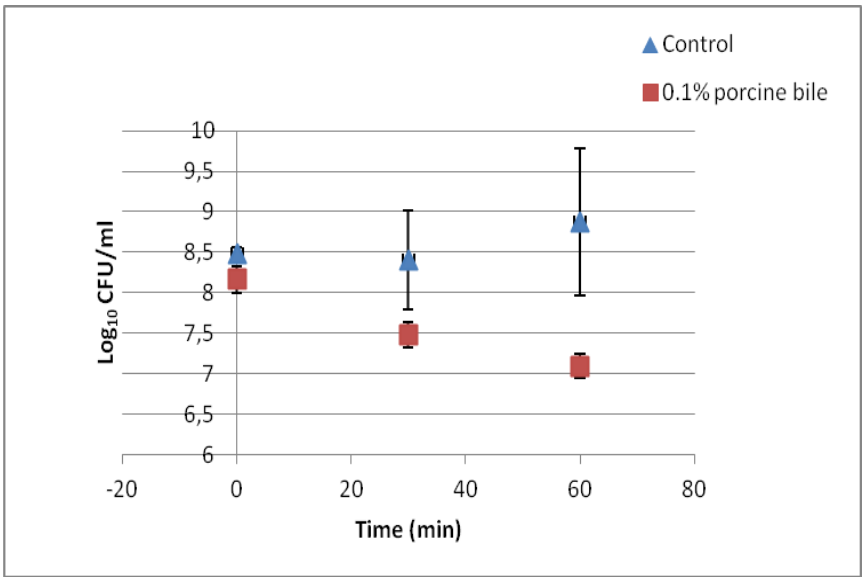

Figure 10: Trend of bacterial survival after bile salts exposure (values are expressed in log_{10} (CFU/ml) ± SD).

3.4.3 Folate content under bile stress

Data on specific folate content at different time points in bile-treated and control samples are shown in figure 9. Regarding the THF content no significant differences were found between treated and control samples at each sampling time, however significant differences could be evidenced between sampling times (0 vs 60, $p<0.05$), showing a peak in THF content at 60 minutes.

$5CH_3H_4$folate was found to be the main folate form both in treated and control samples. Significant differences between treated and untreated cells were found at time 0 and 60 (Figure 9), indeed control samples displayed a greater $5CH_3H_4$folate levels (1434 and 3004 µg/100g dry matter respectively). Also in this case a peak could be registered in $5CH_3H_4$folate content after 60 minutes with significant differences between 0 and 60 both in treated and control samples.

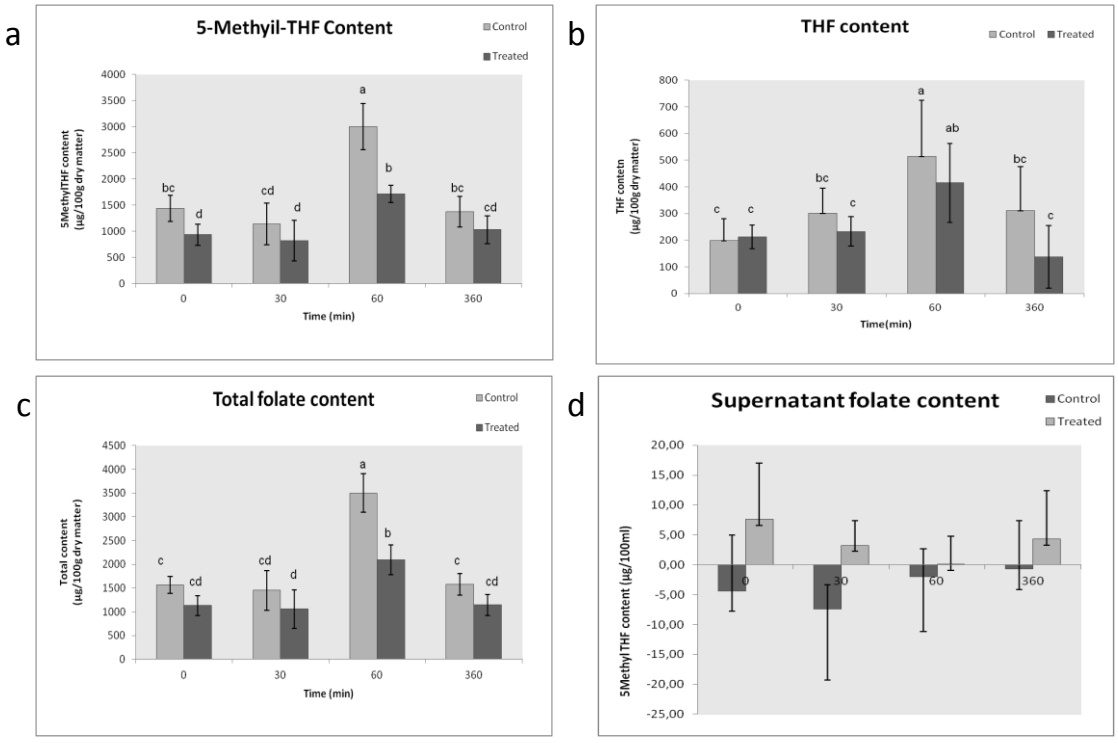

Figure 11: Specific folate content and distribution of main forms in different time points after bile exposure in treated and control cultures: a) 5MehtylTHF content, b)THF content, c) total content, d) Supernatant folate content. Same letter indicates no significant difference, whereas different letters show significant differences (Tukey test $p<0.05$).

The results showed that specific folate content were higher in control cultures. Indeed the folate concentration increased rapidly after one hour and decreased after six hours.

Regarding the extracellular folate levels, we found a different situation (Figure 9d). The only form detected in the supernatant was $5CH_3H_4$folate. Quantities were normalized by subtracting folate levels of the medium. No statistical differences were found between treated and control cells in any time point analyzed.

3.4.2 Gene expression

Gene expression analyses of the 11 genes at different time points are shown in Figure 10.

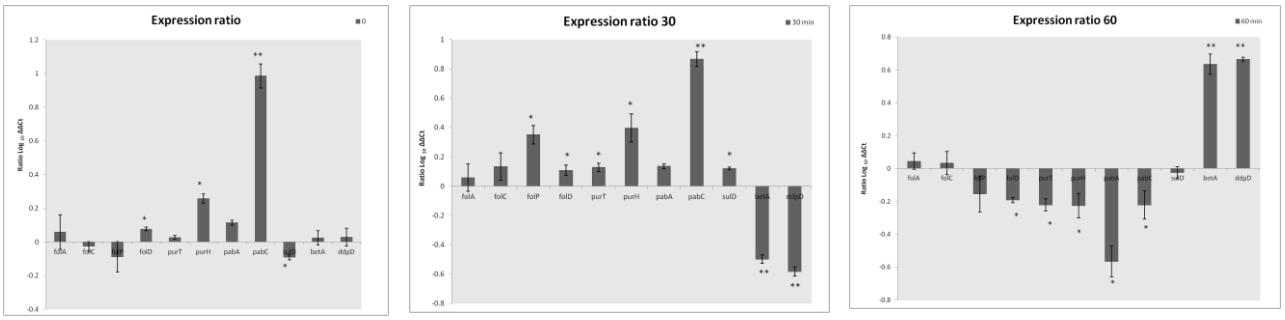

Figure 12: Relative gene expression of bile treated samples in different time point (normalized to the control samples profiles).
*** and ** indicate significativity at p<0.01 and p<0.001 levels respectively.**

At time point 0, immediately after bile exposure, it is possible to note a significant change in the expression profiles of specific genes in treated samples. This finding pointed out the rapid transcriptional response after bile stress (Fig 10a). Expression of *pabC* was significantly (p<0.000) up-regulated, this gene encodes an enzyme for pABA production, a fundamental precursor for folate biosynthesis. On the other hand, the expression of *sulD*, responsible for another folate precursor production (DHPPP), was significantly down-regulated. Other genes changing their expression were *folD* and *purH,* genes belonging to the one carbon pool pathway.

30 minutes after bile inoculation (Fig 10b), 8 genes on 11 changed their expression, at this time also *sulD* is up-regulated, as well as *folP*, a gene encoding for a dihydropteroate synthase, involved in the condensation between pABA and DHPPP. Genes belonging to the one carbon pool pathway (*folD, purT, purH*) were significantly up-regulated (p<0.005). *betA* and *dppD*, encoding respectively for an efflux transporter protein and a transporter ATP-binding protein, were down-regulated (p<0.000).

60 minutes after bile inoculation (Fig 10c), treated cells showed a global down-regulation of folate biosynthesis and one carbon pool pathways. At this time point *betA* and *dppD* genes were significantly up-regulated (p<0.000).

4. Discussion

Bifidobacteria may show a high folate content per unit biomass as reported by D'Aimmo et al. (2012), nevertheless no studies have yet addressed questions regarding the correlation between folate content and gene expression as well as the real folate production in GI conditions.

In this work, in order to go one step further in the understanding of *Bifidobacterium* spp. folate metabolism, two biological aspects have been considered: cellular folate content and the transcriptional profiles of genes belonging to folate pathway were monitored in different strains, growth phases and under bile-stress condition.

Bifidobacterium adolescentis ATCC 15703^T was chosen as model organism, being a promising species in terms of folate production. The assays were performed in a medium of growth rich in nutrients (MRSc) containing also small amounts of folate. This medium choice was done considering that ecological niche of bifidobacteria (GI) is a nutrient-rich environment, and considering that starvation could influence transcriptional profiles.

The first assay aimed to analyze *B. adolescentis* ATCC 15703^T folate levels in different growth phases. Results confirm that intracellular folate content is associated with growth rate (Lin, 2000; Pompei et al., 2007a). A constant increase of intracellular $5CH_3H_4$folate content was evidenced, with a peak during the late exponential phase (Figure 2 and 4). On the other hand the extracellular folate content monitored in our study was really low with slight increase in the death phase. These data are in contrast to Pompei's studies on bifidobacteria strains, that reported a prevalence of extracellular folate accumulation (ranged between 62-91% of total folate production)(Pompei et al., 2007a). Nevertheless some differences in experimental setting could justify the discrepancy monitored, the previous studies were performed on batch cultures using a microbiological assay.

Relative expression analysis of *B. adolescentis* ATCC 15703^T genes in different growth phases (normalized for the expression level OD 0.3) evidenced a down-regulation of the pathway with folate accumulation. Genes involved in folate precursor production (*sulD* and *pabC*) were down-regulated in both growth phases (0,6 and 0,9 vs 0.3). Probably, the high folate content already reached at OD 0.6 does not require the production of additional amounts of pABA and DHPPP. Another gene down regulated, both in OD 0.6 and 0.9, is *purH*, a gene of the one carbon pool pathway transforming THF in 10-formylTHF.

Five different strains of *B. adolescentis* (ATCC 15703^T, B7311, MB21, F365, B7162) were screened for their ability to produce folates and significant differences were found, revealing B7311 and B7162 as the two highest folate producing strains. Folate content is therefore strain-dependent, but all the *B. adolescentis* strains analyzed in this study are able to produce remarkable folate levels. Considering

the large distribution of *B. adolescentis* in the human adult microbiota, member of this species could significantly contribute to human folate intake.

Comparing relative expression levels of strains ATCC 15703^T and B7311 no significant differences could be observed that can explain the variation in folate content. The global expression patterns in B7311 did not show clear relationship with its overproduction of folates at the studied sampling times. Neverthless the global pictures of expression patterns in B7311 revealed a clear downregulation of a folate precursor gene, *pabC*, suggesting a suspension of folate precursor production in presence of high amounts of folates.

It is possible to suppose that folate accumulation and differences in transcriptional levels rely on the early growth phases.

An important key point of this work was to investigate the influence of bile on folate metabolism, being bile exposure a relevant stress that bifidobacteria daily face, with impact on global metabolic pathway. In order to get closer to bile condition of the human colon, cultures of ATCC 15703^T were treated with 0.1% porcine bile. Despite the low bile concentration, results evidenced a significant influence on *B. adolescentis* survival, gene expression and folate production after bile exposure.

Viable microbial counts revealed differences between control and bile-treated cultures, (with a log difference after 1 hour of exposure) actual demonstrating the bactericidal effects of bile and the relative influence on bifidobacterial survival. Surviving cells, as reported by Ruiz et al. (2012) and Sanchez et al. (2005), respond with a metabolic reorganization in order to minimize bile-induced damages, thus modifying their gene expression profile.

Transcriptional analysis showed a dynamic reply through different time points. Already at the sampling time zero significant differences were monitored, indicating a rapid transcriptional response. In particular *pabC* was significantly up regulated, suggesting an over-production of pABA. Genes involved in the folate biosynthesis as well as genes of the one carbon pool pathway were also up-regulated after 30 minutes of bile exposure. A possible explanation for the activation of folate biosynthesis pathway after bile exposure might be related to the bile effect on DNA, indeed it is well-known that bile induces DNA damage in microorganisms and bifidobacteria respond with the activation of enzymes involved in DNA repair (insert reference). Being folate responsible for the biosynthesis of purines, and thus for nucleotides synthesis, the folate pathway activation could be required for DNA repair. 60 minutes after bile inoculation, treated cells showed a global down-regulation of folate pathways. On the other hand the positive control *betA* as well as *dppD* genes, encoding for an efflux transporter protein and a transporter ATP-binding protein respectively were strongly activated. Probably, this long exposition to bile stress induce the activation of mechanisms to neutralize bile toxicity, such as an active extrusion of bile salts, reflected in an over-expression of these two genes. In summary, bifidobacterial cells react with a first attempt to contrast the bile

effects (T 30 min), which however is not sufficient to stem damages, followed to an active extrusion of bile salts (T 60 min). Folate quantification showed significant differences between control and bile-treated cultures concerning both total folate content, as well as $5CH_3H_4$folate and H_4folate. Control samples displayed higher levels of both forms of folate after 60 minutes, confirming the bile effect on folate metabolism. Despite the over-expression of folate pathways, vitamin B9 levels in treated cells were lower than control, suggesting a possible over-consumption of folate by stressed cells or an extrusion of folate in the extracellular environment. However the supernatant analysis didn't reveal an increase of free folate. Our data suggest that bile exposure influence bifidobacterial survival and folate metabolism.

4. Conclusions

The selection and use of folate producing microorganisms could be an innovative strategy to increase human natural folate levels (Leblanc et al., 2007), and for this reason a comprehensive analysis on *Bifidobacterium* folate metabolism could be useful to understand the feasibility of the strategy. This work provided a global analysis on folate production and gene expression of *Bifidobacterium adolescentis* strains. Our work revealed that folate production is strictly correlated with growth phases and it is strain-specific, however all analyzed strains showed a high folate content. *Bifidobacterium adolescentis* reveals itself as a promising species in terms of folate biosynthesis and his aspect represents an added value in the selection of probiotic *B. adolescentis* strains. The concentration of folate in *B. adolescentis* was really higher in respect to cell requirement and no clear reasons are available; the simplest explanation could rely in the bacteria-host symbiotic relationship. Furthermore, in the perspective to develop a probiotic product containing folate producing strains, the evaluation of the impact of the gastrointestinal environment on folate metabolism could be of importance. For this reason bile effect on folate metabolism was analyzed demonstrating that bile exposure negatively influences cell survival as well as folate metabolism. In this perspective an evaluation of bile tolerance could be useful for the selection of folate producing strains. Considering their abundance in human microbiota, *Bifidobacterium adolescentis* strains may represent an endogenous source of natural folate and they could be used to fortify fermented dairy products.

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Conclusion

The work reported in this thesis gives a fundamental understanding of folate metabolism in *Bifidobacterium adolescentis.* This species was chosen as model organism for this study, considering its folate related characteristics, that make it a promising species in terms of vitamin B9 production. The work was subdivided in three main phases, also aimed to solve different problems encountered working with *Bifidobacterium* strains.

The first goal of this PhD thesis was to design a new identification method, able to rapidly discriminate *Bifidobacterium* species. The developed protocol was based on the restriction endonuclease analysis of the PCR-amplified *hsp60* gene sequence (*hsp60* PCR-RFLP) using a single restriction enzyme (*HaeIII*). This method represents a rapid, reproducible, inexpensive and easy-to-handle molecular tool for the identification of *Bifidobacterium* species isolated from various environments. A total of 37 *Bifidobacterium* species and subspecies were analyzed with this technique, which give species-specific fingerprinting, allowing an efficient discrimination also for very closely related species and subspecies.

Moreover the *hsp60* PCR-RFLP technique was validated by testing numerous strains for each species confirming the conservation of the profiles concerning the species and subspecies tested.

This research led to the publication of two papers:

- "Identification of species belonging to the *Bifidobacterium* genus by PCR-RFLP analysis of a *hsp60* gene fragment" Baffoni et al., (2013) on BMC Journal;
- "Identification of *Bifidobacterium* spp. using *hsp60* PCR-RFLP analysis: an update" Stenico et al., (2014) on Anaerobe;

The second achievement concerns the evaluation of *Bifidobacterium adolescentis* biodiversity (Paper 3). Different aspects were assessed in order to have a deep insight into the intra-species variability and to identify representing strains of this species to be screened for their folate production ability. Investigations included a multilocus approach, the use of algorithm for ecotype demarcation and

different fingerprinting methods. The research showed that *Bifidobacterium adolescentis* species is characterized by a wide phenotypic and genetic variability, and the HMFA analysis led to the identification of 8 clusters to describe all the biodiversity. Clusters allowed the selection of representative strains for the further work on folates. The outcomes obtained with this research supported the idea that this species would require a new taxonomic re-organization. Moreover the type strain of *B. adolescentis* ATCC 15703^T is defined by nonconforming characteristics; and placed itself at a significant distance to any other analyzed strain in all the cluster analyses performed. Therefore a broad number of strains would be suitable to correctly describe the real common characteristics and properties of a species.

This work led to the preparation of one paper:

- "Looking inside the intra-species diversity of *Bifidobacterium adolescentis*" Stenico et al., (2014) under submission;

An overview of *Bifidobacterium adolescentis* folate metabolism was another goal achieved in this thesis. This work provided a global analysis on folate production and gene expression of *Bifidobacterium adolescentis* strains, using a validate HPLC method and qRT-PCR technique.

The first step was to validate appropriate reference genes for the qRT-PCR normalization, that represents an essential prerequisite for producing accurate real-time PCR expression analyses. Two different algorithms (BestKeeper and NormFinder) were used to evaluate the stability of 9 putative reference genes. This research is the first attempt to validate reference genes in *Bifidobacterium* spp. and the results offer an appropriate set of reference genes suitable for qRT-PCR studies on *Bifidobacterium adolescentis* strains. This work led to the publication of one paper:

- "Validation of candidate reference genes in *Bifidobacterium adolescentis* for gene expression normalization" Stenico et al., (2014) on Anaerobe;

HPLC quantification (performed at Chalmers University of Technology, Goteborg, SW) showed that the form of folate mainly found in *B. adolescentis* was $5CH_3H_4$folate, followed by H_4folate. Variations

in specific folate content were observed in the analyzed *B. adolescentis* strains (selected considering the previous cluster evaluation), confirming its wide intrinsic intra-species variability. Remarkable folates levels were found in all strains under investigation. In addition the research evidenced that the 5-CH_3H_4folate content increased with increasing growth rate, suggesting that folate production is strictly correlated with *B. adolescentis* growth phases. Relative gene expression analysis evidenced both a down-regulation of the folate pathway and precursor production with folate accumulation.

A further key point of this work was to investigate the influence of bile on folate metabolism, being bile exposure a relevant stress that bifidobacteria daily face. We reported that bile exposure has a strong influence on bifidobacteria metabolism, significantly affecting bacterial survival, folate content, as well as relative gene expression. Surviving bile treated cells displayed lower levels of folates (with HPLC quantification), suggesting that cells consumed this vitamin to face bile damages. Relative gene expression analysis seemed to indicate that bifidobacterial cells react with a first attempt to contrast the DNA damage caused by bile, with an activation of the folate pathway, followed to an active extrusion of bile salts.

This work led to the preparation of one paper:

- "Gene expression and folate production in *Bifidobacterium adolescentis* strains" Stenico et al. (2014) under submission;

The selection and use of folate producing microorganisms represents an innovative strategy to increase human folate levels. *Bifidobacterium adolescentis* strains may represent an endogenous source of natural folate and they could be used to fortify fermented dairy products. This bio-fortification strategy presents many advantages for the consumer, providing natural folate forms more bio-available than folic acid, the form used in fortification program. These native forms are not implicate in the discussed controversy concerning the safety of high intake of synthetic folic acid. However the addition of probiotics in food products requires to take into account several aspects, as for example shelf-life and gut persistence. In this context the evaluation of the stress effects, both on survival and on folate production performance, is essential. An attempt to circumvent these problems could be represented by the administration of *B. adolescentis* cells lysates, that could constitute a dietary supplement able

to meet folate intake recommendations, avoiding the consumption of synthetic form. Further research concerning the administration of bacterial cells lysates are encouraged.

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