

Bifidobacteria and lactobacilli in the gut microbiome of children with non-alcoholic fatty liver disease: which strains act as health players?

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Abstract

Introduction: Non-alcoholic fatty liver disease (NAFLD), considered the leading cause of chronic liver disease in children, can often progress from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH). It is clear that obesity is one of the main risk factors involved in NAFLD pathogenesis, even if specific mechanisms have yet to be elucidated. We investigated the distribution of intestinal bifidobacteria and lactobacilli in the stools of four groups of children: obese, obese with NAFL, obese with NASH, and healthy, age-matched controls (CTRLs).

Material and methods: Sixty-one obese, NAFL and NASH children and 54 CTRLs were enrolled in the study. Anthropometric and metabolic parameters were measured for all subjects. All children with suspected NASH underwent liver biopsy. Bifidobacteria and lactobacilli were analysed in children’s faecal samples, during a broader, 16S rRNA-based pyrosequencing analysis of the gut microbiome.

Results: Three *Bifidobacterium* spp. (*Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium adolescentis*) and five *Lactobacillus* spp. (*L. zeae*, *L. vaginalis*, *L. brevis*, *L. ruminis*, and *L. mucosae*) frequently recurred in metagenomic analyses. *Lactobacillus* spp. increased in NAFL, NASH, or obese children compared to CTRLs. Particularly, *L. mucosae* was significantly higher in obese ($p = 0.02426$), NAFLD ($p = 0.01313$) and NASH ($p = 0.01079$) than in CTRLs. In contrast, *Bifidobacterium* spp. were more abundant in CTRLs, suggesting a protective and beneficial role of these microorganisms against the aforementioned diseases.

Conclusions: Bifidobacteria seem to have a protective role against the development of NAFLD and obesity, highlighting their possible use in developing novel, targeted and effective probiotics.

Key words: paediatric non-alcoholic fatty liver disease, gut microbiota, *Bifidobacterium*, *Lactobacillus*.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in adults and children. It is regarded as a spectrum of hepatic conditions, which ranges from steatosis (non-alcoholic fatty liver – NAFL) to non-alcoholic steatohepatitis (NASH), with or without fibrosis [1]. The pathogenesis of NAFLD has been partially elucidated. It is generally accepted that NAFLD is pathogenically a “multiple-hit” disease. This hypothesis focuses on the central role of elevated circulating levels of free fatty acids (FFA) and insulin resistance that mutually interact to cause FFA accumulation in hepatocytes, oxidative stress and an inflammatory response, providing the grounds for progressive liver damage [2]. Interestingly, several studies have shown that gut microbiota may also play a key role in NAFLD development and progression [1]. Microbiota refers to the ecological community of commensals, symbiotic and pathogenic microorganisms that share our body space and internal cavities. The human microbiota consists of about 100 trillion microbial cells, outnumbering human cells 100 to 1 [3], with five phyla representing the majority of the community. There are approximately 160 species in the large intestine of any individual, and very few of these are shared between unrelated individuals. It has been demonstrated that microbiota composition can influence the proportion of calories absorbed from intestinal contents [4].

Indeed, bacteria play an important role in the absorption and emulsion of fat and fat-soluble vitamins in the small bowel. The gut microbiota may also produce endotoxins, such as lipopolysaccharide (LPS), that during impaired intestinal permeability may reach the circulation, leading to extra- and intra-hepatic inflammatory responses, which, in turn, induce the progression from NAFL to NASH [1]. It is now recognised that the gut microbiota and chronic liver disease are closely linked. In addition, Ridaura *et al.* demonstrated a potential relationship between the gut microbiome and the development of an obese phenotype [5].

Recently, our group demonstrated that omics-based integrated charts of gut microbiota may profoundly assist in the understanding of correlations between enterogradients and NAFLD onset and progression, thus suggesting either phylome or metabolome indicators that may be used as phenome biomarkers of gut microbiota disease [6].

Furthermore, the gut microbiota is influenced by diet, age, infection, hygiene habits and antibiotic therapy [7]. Patients affected by NAFLD showed a high prevalence of small intestine bacterial overgrowth (SIBO) and increased intestinal permeability that improved bacterial translocation, exposing the liver to toxic microbial metabolites

[8, 9]. Obese patients with NAFLD displayed a reduction of Bacteroidetes numbers and an increase in Firmicutes numbers compared to patients with normal weight [10]. Intriguingly, the loss of weight in response to the limited intake of carbohydrates and fats increased the prevalence of Bacteroidetes in the intestinal microbiota [10]. Furthermore, studies on mice with NAFLD have demonstrated that lactobacilli and other probiotic bacteria reduced plasma cholesterol concentrations and protected against fat accumulation in adipose tissue and hepatocytes [8, 11].

Understanding the nature of gut dysbiosis, the integrity of the intestinal barrier and mechanisms of the hepatic immune response to gut-derived factors is potentially relevant to the development of new therapies to treat chronic liver diseases [12]. The term “dysbiosis” was coined by Metchnikoff to describe altered pathogenic bacteria in the gut [13]. Dysbiosis was defined as qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution [14]. Recently, we demonstrated [15] that probiotic treatment in obese children with NAFLD reduced their body mass index (BMI) and hepatic steatosis as assessed by ultrasound, and increased circulating levels of glucagon-like peptide-1 (GLP-1), a potent hormone released from intestinal L cells [16]. These encouraging results, also confirmed by other studies [17–19], suggest that improving our knowledge of bacterial species that live in the gut of NAFLD subjects may contribute significantly to allowing us to select the best probiotic(s) to strongly ameliorate liver damage.

In a recent, extensive characterisation of the gut microbiota profile of children with NAFLD compared to obese and lean children without liver disease, it emerged that *Oscillospira* is a marker of the healthy status of the liver, while *Ruminococcus* and *Dorea* increased concomitantly with NAFL towards NASH progression [6]. However, since the most commercially available probiotics are *Bifidobacterium* and *Lactobacillus*, the aim of the present study was to discuss these species in terms of the severity of disease in order to determine which can be used as treatment for children with NASH.

Material and methods

This study was conducted on 61 consecutive overweight or obese child patients (35 males, aged 11.45 ± 2.42 years) who were followed at the Hepato-Metabolic Disease Unit of Bambino Gesù Children’s Hospital and Research Institute (OPBG; Rome, Italy) in 2013. All patients were tested for secondary causes of fatty liver such as hepatitis, cytomegalovirus and Epstein-Barr viral infections, liver disease, autoimmune disease, metabolic liver disease, Wilson’s disease, coeliac disease or a de-

iciency of α_1 -antitrypsin, which were excluded using standard clinical, laboratory and histological criteria. Fifty-three patients, with BMI > 95th percentile, and/or hypertransaminasaemia and steatosis, underwent a liver biopsy for the evaluation of liver damage. The study population consisted of three groups: 8 (13.12%) obese, 27 (44.26%) with NAFLD and 26 (42.62%) with definite NASH. Moreover, 54 healthy, age-matched children enrolled at the OPBG Human Microbiome Unit were used as controls (CTRLs).

The Hospital Ethics Research Committee approved the study, which was conducted in accordance with the Declaration of Helsinki (as revised in Seoul, Korea, October 2008). Written informed consent was obtained from the parents of the enrolled subjects. This study was approved by the OPBG Ethics Committee (protocol no. 768.12).

Faecal samples were collected from all affected children and CTRLs, and analysed at the OPBG Human Microbiome Unit.

Anthropometric and laboratory evaluations

Anthropometric parameters, including weight, height, BMI, and waist circumference, were measured in all children using standardised methods. Obesity was defined by a BMI \geq 95th percentile, adjusted for age and sex according to World Health Organization growth charts. Fasting plasma glucose (FPG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ -glutamyltransferase (GGT), fasting serum triglycerides (TG), and total cholesterol were determined using standardised methods in our laboratory. Insulin was measured by means of a radioimmunoassay (MYRIA; Technogenetics, Milan, Italy). The degree of insulin sensitivity/resistance was determined by a homeostatic model assessment (HOMA). A HOMA value > 2 was considered an indication of insulin resistance [20]. All patients underwent an abdominal ultrasound evaluation. Hepatic ultrasonography scanning by assigned and experienced radiologists, who were blinded to the health conditions of the patients, was performed on children after overnight fasting. The liver was graded for markers of hepatic steatosis by using established criteria: a bright hepatic echo pattern (compared with the echo response of the right kidney). All patient data have been previously reported by Del Chierico *et al.* (Table I from [6]).

DNA extraction

Every stool sample collected from the 115 children was processed for further 16S rRNA-targeted metagenomic analysis. From each sample, DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany). To start, stool sam-

ples were resuspended in 1.5 ml of phosphate-buffered saline, homogenised by vortexing for 2 min and centrifuged at 20,800 \times g. After the removal of supernatant, the pellet was resuspended in 500 μ l of phosphate-buffered saline, together with 500 μ l of beads/PBS (1 mg/ μ l, [w/v]; glass beads, acid-washed Sigma-Aldrich, St. Louis, MI, USA). The supernatant was collected and treated for one freeze-thaw cycle ($-20^{\circ}\text{C}/70^{\circ}\text{C}$) for 20 min each step, as previously described. After centrifugation at 5200 \times g for 5 min, the supernatant was subjected to QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) extraction according to the manufacturer's instructions and then processed for metagenomics pipelines, as previously described [15]. The gut microbiome was investigated by pyrosequencing V1-V3 regions of the 16S rRNA gene (amplicon size 520 bp) on a GS Junior platform (454 Life Sciences, Roche Diagnostics, Monza, Italy), according to the original pipelines described by Del Chierico *et al.* 2016 [6].

Statistical analysis

Univariate testing for differential abundances of each taxonomic unit between two or more groups was tested using a non-parametric Kruskal-Wallis H test.

Open access data repository

Bioprojects healthy children gut microbiota: PRJNA268064, PRJNA 268067, PRJNA268144, PRJNA268157, PRJNA268286 and PRJNA280490, Metaomic OPBG Consortium (<http://www.ncbi.nlm.nih.gov/bioproject/?term=>).

Results

Our observation, based on the study results reported previously [6], highlighted the prevalent distribution of three main *Bifidobacterium* spp. (*B. longum*, *B. bifidum*, and *B. adolescentis*) and five *Lactobacillus* spp. (*L. zeae*, *L. vaginalis*, *L. brevis*, *L. ruminis*, and *L. mucosae*) that variously recurred in targeted metagenomic analyses. In particular, *L. zeae* was present in all four groups (i.e., obese, NAFL and NASH children), while *L. ruminis* and *L. vaginalis* were present only in the NAFL and NASH groups. *Lactobacillus mucosae* was associated with obese and NAFL groups, and *L. brevis* with NASH and CTRL groups (Figure 1). Interestingly, Kruskal-Wallis H test performed among the groups revealed that *L. mucosae* was significantly higher in obese ($p = 0.02426$), NAFLD ($p = 0.01313$) and NASH ($p = 0.01079$) than in CTRLs.

In contrast, an inverse correlation between bifidobacteria and NAFL and NASH was observed; in particular, while *B. longum*, *B. adolescentis* and *B. bifidum* were shown to be strongly reduced in

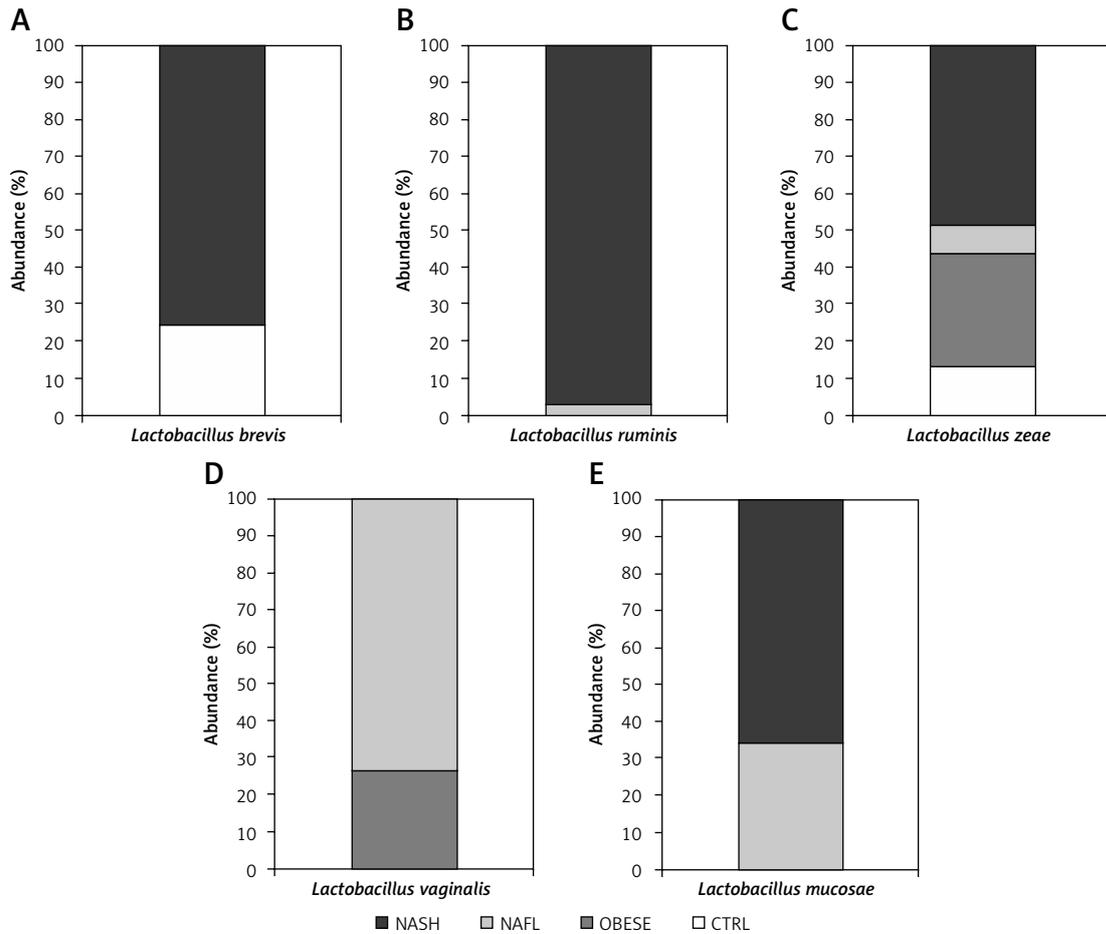


Figure 1. Histograms showing the percentage of relative abundances of *Lactobacillus* spp. distributed among the various groups of children. Abundances were calculated for: **A** – *L. brevis*; **B** – *L. ruminis*; **C** – *L. zeae*; **D** – *L. vaginalis* and **E** – *L. mucosae*

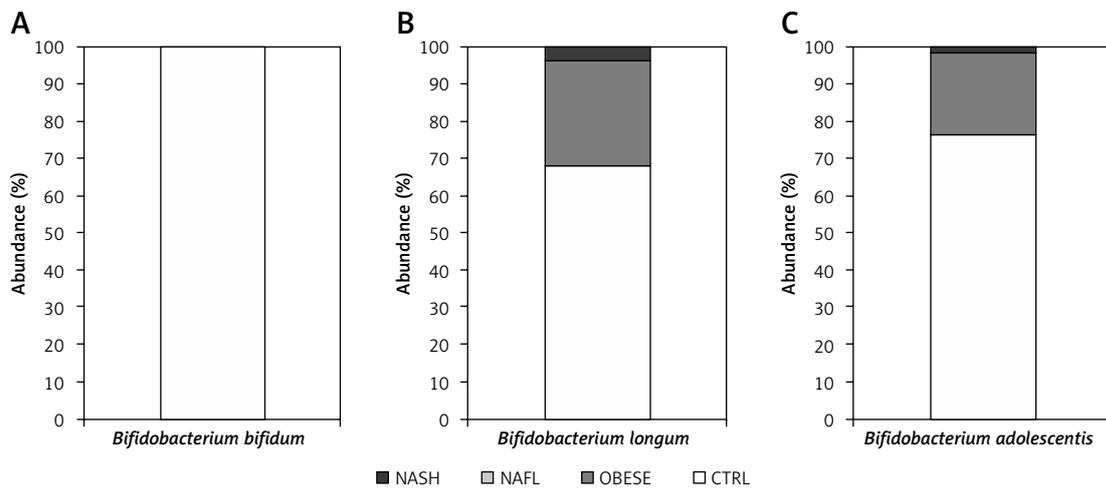


Figure 2. Histograms showing the percentage of relative abundances of *Bifidobacterium* spp. distributed among the various groups of children. Abundances were calculated for: **A** – *B. bifidum*; **B** – *B. longum*; and **C** – *B. adolescentis*

number in all patients, they were present in much higher levels in CTRLs. In fact, *B. bifidum* was present only in the CTRL group, while *B. adolescentis* and *B. longum* were both present in high numbers in CTRLs, and at low levels in obese and NASH groups (Figure 2).

Discussion

In recent years, scientific and pharmaceutical researchers have focused their efforts on developing probiotic strains for the therapy of several diseases, since scientific evidence has shown an association

between a specific probiotic strain with each disorder [21–31]. Recently, new studies in systems biology have substantially supported the selection of specific, disease-targeted probiotics [6, 32].

In previous studies, bifidobacteria were able to decrease the NAFLD activity score by reducing hepatic exposure to intestinal products such as LPS that promote hepatic inflammatory cytokine production and oxidative stress, leading to liver injury [33, 34]. *Bifidobacterium longum* as a probiotic has been observed to reduce endotoxin levels, thus promoting reduced hepatocellular injury, portal and parenchymal inflammation, and NASH activity [34]. Ren *et al.* highlighted the ability of bifidobacteria to increase the hepatic expression of sirtuin 1 α (SIRT1- α), which, in turn, up-regulated the expression of the peroxisome proliferator-activated receptor α (PPAR- α) and down-regulated the level of the sterol regulatory element-binding protein 1c (SREBP-1c), thus preventing the progression of NAFLD [35].

In the present study, we also observed an increase of *Lactobacillus* spp. in NAFL and NASH patients compared to CTRLs. These data contrast with a previous experimental study demonstrating that the administration of a *L. johnsonii*-based probiotic to mice fed a high-fat diet (HFD) was able to prevent the development of NAFLD [35]. More specifically, the probiotic supplementation promoted an increase of lactobacilli and a decrease of bacteria from the Enterobacteriaceae family in the gut, providing preliminary protection. Moreover, it reduced both gut permeability and LPS serum levels, which are generally altered in mice with HFD-induced NAFLD. It is worth noting that dysfunctional gut permeability may allow liver exposure to gut-derived bacterial metabolites or antigens [36]. Similarly, gut microbiota-derived LPS leads to liver inflammation and oxidative stress [37]. Interestingly, Sohn *et al.* demonstrated that administering *L. paracasei* may prevent an increase in intestinal permeability in an NASH model by inhibiting the pro-inflammatory M1 Kupffer cell response and promoting the anti-inflammatory M2 response [38]. The discrepancy between these results and our study may be attributable to strain-specific activity of lactobacilli or to the use of animal models that may not be fully representative of human physiology.

It is noteworthy that all obese patients enrolled in the present study showed a significant decrease in bifidobacteria abundance compared to CTRLs, thus also suggesting a possible role for bifidobacteria in this disease. Indeed, their role in obesity is controversial, some studies demonstrating an involvement in body weight gain, with others showing opposite results [39, 40]. In particular, *B. longum* has been observed to significantly reduce body weight gain and fat weight in a group of mice fed for 5 weeks with a HFD [39]. Interest-

Bifidobacteria and lactobacilli: good and bad for NAFLD	
	GOOD/BAD
<i>Lactobacillus brevis</i>	Red
<i>Lactobacillus ruminis</i>	Red
<i>Lactobacillus zeae</i>	Red
<i>Lactobacillus mucosae</i>	Red
<i>Lactobacillus vaginalis</i>	Red
<i>Bifidobacterium bifidum</i>	Blue
<i>Bifidobacterium longum</i>	Blue
<i>Bifidobacterium adolescentis</i>	Blue

Figure 3. Schematic representation of the beneficial and unfavourable effects of bifidobacteria and lactobacilli in paediatric NAFLD

ingly, the anti-obesity activity of probiotic microorganisms is often associated with a hypocholesterolaemic effect, through inhibition of cholesterol synthesis, elimination of cholesterol in faeces, absorption of cholesterol back into the body and inhibition of bile salt recycling [41, 42].

Lactobacillus spp. are associated both with normal weight and obesity; however, it is conceivable that these species, often found in the gut of obese subjects, may share with *L. reuteri* the same mechanism underlying weight gain: improvement of the intestine's ability to absorb and process nutrients, which increases food conversion and finally leads to body weight gain [43]. The hypocholesterolaemic properties of lactobacilli have already been reported. In fact, these bacteria increased high-density lipoprotein in serum and short-chain fatty acid (SCFA) concentrations in faeces, and promoted faecal bile acid excretion [44]. Moreover, some *Lactobacillus* spp. may exhibit antidiabetic activity, with a significant reduction of blood glucose levels seen in infected mice [37, 45].

In conclusion, we highlight that bifidobacteria and lactobacilli strains may differently impact NAFLD (Figure 3). Noticeably, we highlight that some *Bifidobacterium* spp. may have a protective role in the development of NASH, NAFL and obesity due to their marked reduction in patients with these disorders. Further studies should focus on assessing whether the administration of specific and well-selected *B. adolescentis*, *B. longum* and *B. bifidum* strains, alone or in combination with *Oscillospira* [6], may improve symptoms and the clinical course of NAFLD in obese children.

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Conflict of interest

The authors declare no conflict of interest.

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