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Bifidobacterium breve M4A and *Bifidobacterium longum* subsps. *longum* FA1 Reduced Weight Gain and Hepatic Lipid Droplets in Young Mice Fed High-Fat

Mustafa Alsharafani[1]*, Martin Roderfeld[2], Elke Roeb[2] and Michael Krawinkel[1]

[1]*Institute of Nutritional Sciences, Justus Liebig University, Wilhelm Str, Giessen, Germany*
[2]*Department of Gastroenterology, Uniklinikum Giessen and Marburg GmbH, Centre for Internal Medicine, Justus Liebig University, Klinik Str, Giessen, Germany*

Abstract

Among commensal bacteria, Bifidobacteria (*B*) is one of the most numerous probiotics in the mammalian gut. The goal of the study was to evaluate the *in vivo* anti-obesity effect of *B. breve* M4A and *B. longum* subsp. *longum* FA1 in young mice fed a high-fat diet (HFD). Three (male mice C57BL/6JRj) groups, the model HFD group and treatment (HFD-FA1 and HFD-M4A) groups were fed an HFD to induce obesity. After feeding the mice a HFD for six weeks, animals receiving *B. breve* M4A and *B. longum* subsp. *longum* FA1 had significantly lower ($p<0.01$) weight gain compared to mice fed a high-fat diet only. Mice fed *B. breve* M4A supplemented with 0.3% yeast extract and 3% glucose exhibited significantly lower serum triglycerides ($p<0.05$) compared with the HFD group. The daily consumption (2.9×10^6 CFU/day) of *B. longum* subps. *longum* FA1 and (4.1×10^6 CFU/day) *B. breve* M4A ($p<0.01$) significantly increased the amount of bifidobacteria and lactic acid bacteria in the cecal content. This study showed that *Bifidobacterium* species reduced weight gain and hepatitis lipid droplets. Thus, bifidobacteria supplementation may be one mean for reducing obesity and related chronic Non-communicable diseases.

Keywords: Bifidobacteria; High fat diet; Triglyceride; Body weight

Introduction

The gut microbiota contributes to the host response towards nutrients [1]. *Bifidobacterium* species are common inhabitants of the human gut intestinal tract of human, different animals and sewage [2,3]. In human some bifidobacteria are commensal or health-promoting, also classified as probiotic microorganism like *B. bifidum* and *B. breve* [4-6]. The gut microbiota contributes to the host response towards nutrients [1,7]. It has been proposed that alterations in the composition of the gut microbiota in the triglyceride accumulation and hypercholesterolemia, and that participated in obesity [8]. The development of obesity and type 2 diabetes following feeding a high-fat diet was characterized by specific changes of the bacterial populations which were predominant in the gut microbiota. In a previous study, *B. longum* showed a more significant effect in lowering serum total cholesterol than a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subps. *bulgaricus* both in rats and humans. These results suggested that some specific strains of bifidobacteria linked to lipid metabolism and body weight may be potentially therapeutic when applied for managing obesity [9]. Attempts have been made to increase the number of bifidobacteria in the intestinal tract by supplying specific bifidobacteria strains and foodstuffs that stimulate the growth of Bifidobacteria [10,11]. Yin et al. concluded that the response of energy metabolism to bifidobacteria was strain-dependent [12]. Therefore, different strains of bifidobacteria might lead to different fat distributions. The overall aim of this study was to evaluate the anti-obesity activity of Bifidobacteria strains grown in skimmed milk media on young mice fed a high-fat diet.

Methods

Bifidobacteria strains

Two *B. breve* M4A and *B. longum* subsp. *longum* FA1 were used that were isolated and identified from the feces of the healthy breastfed infants [13]. Identification of *Bifidobacteria* was based on phenotypic traits and molecular-based methods as amplification and sequencing of the 16S RNA gene [14]. The isolates were conserved by freeze-drying until use. The colonies were enumerated and recorded as colony forming units per milliliter from lyophilized strains by added 1 mL sterilized distilled water into each lyophilized vial for 30 min. The results were arithmetic means of three measurements.

Experimental design

The relationship were evaluated between Medium (yeast extract, oligofructose and glucose) on pH via count number as a mediator using multiple, nonlinear regression models. Multiple Regression analyses of dependent factors of pH value and count number [13]. In order to obtain the desired number of bifidobacterium strains, the medium was supplemented with carbohydrates to enhance its growth and survival. Skimmed milk powder containing 10% solids (Merck) was reconstituted in distilled water. Skimmed milk base (containing 0.3% yeast extract) was supplemented with 3% (*w/v*) oligofructose for culture of *B. longum* subsp. *longum* FA1 and 3% glucose for *B. breve* M4A. The media were separated in capped bottles containing 10 mL media, flashed with CO_2/N_2 gas into bottles and sterilised by autoclaving at 121°C for 10 min, and inoculated with 10% (*v/v*) *B. longum* subsp. *longum* FA1 or *B. breve* M4A cultures after the activation of each pure lyophilised culture for 24 h. The cultures were incubated anaerobically at 37°C, and the pH was adjusted: ~ 5 mL samples were withdrawn every 16 h for analysis. A single dose of 0.20 (mL/day) was administered orally by gavage to each mouse. The fermented milk cultures administered to the HFD-FA1 and HFD-M4A groups contained 2.9×10^6 (CFU/day) of *B. longum* subsp. *longum* FA1 and 4.1×10^6 (CFU/day) of *B. breve* M4A, respectively. For establishing an equal stress, the HFD group was orally (by garage) given the same dose skimmed milk supplemented with 0.3% yeast extract and 3% glucose as the intervention groups.

***Corresponding author:** Mustafa Alsharafani, Institute of Nutritional Sciences, Justus Liebig University, Wilhelm Str, 20/35392 Giessen, Germany, Tel: +490641/9939035; Fax: +490641/9939039; E-mail: mustafa.email@yahoo.com

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During the first week of the study, mice in the high fat diet *B. longum* subsp. *longum* FA1 (HFD-FA1) and high fat diet *B. breve* M4A (HFD-M4A) groups were given a simple control diet for adaptation. From week 2 to 6 both groups were supplemented with *B. breve* M4A and *B. longum* subsp. *longum* FA1, administered by gavage orally. A third group received a high fat diet only (HFD).

The interaction between body composition and nutrition differs among mouse species. In addition, different diets elicit different reactions towards obesity among mice [15]. The effects expected in the current study were estimated according to Kondo et al. who performed a study for >8 weeks, but detected effects after six weeks. The minimum number of mice was calculated (1) based on a significant difference in epididymal fat pad between the control and *Bifidobacterium* 3H groups. (2) Significant differences in weight gain between the control and B-3H groups were identified after six weeks [16]. The alpha error was set at 0.05 (two-sided), and the tolerated beta error was 0.2. No correction for multiple comparisons was performed, and calculations were based on parametric tests (one-way ANOVA with LSD post hoc test). The calculations were performed using '3.1 GPower©' (University of Duesseldorf, Germany). Six animals per group were needed for a minimum sample size based on the above assumptions. In addition, variance heterogeneity might result in higher scatter of the measured values. Therefore, we increased the number of mice per group to seven.

Animal experiments

The study was achieved in strict accordance with the recommendations in the guide for the Federation of Laboratory Animal Science Associations (FELASA) recommendations in specific barrier facilities in the animal care centre of the Institute of Pharmacology and Toxicology, Justus-Liebig-University Giessen. The district president of Giessen approved the study protocol. All experiments were performed under specific pathogen-free conditions. Each cage was closed using a filter top and was ventilated individually with modified barrier-systems (individually ventilated cages-IVC). Cages were only opened under laminar flow conditions at the cage changing station.

Male C57BL/6JRj mice (Janvier, 53941-ST-Berthevin, France) aged six weeks were divided randomly into three groups of seven mice. Mice were maintained at 22 ± 2°C with 60 ± 5% relative humidity, a 12 h light/dark cycle and food ad libitum. All mice were fed the control diet (C1090-10-Altromin-Spezialfutter, Germany) for one week to stabilize their metabolism. The diet contained 10 energy percent fat, 71% carbohydrates, and 19% protein. The control and treatment groups were fed a high-fat diet (C1090-70-Altromin-Spezialfutter, Germany) to induce obesity for six weeks. In the high-fat diet 70% of energy were derived from fat, 15% from carbohydrates, and 15% from protein. Table 1 indicates the principle composition of both diets.

The drinking water of mice in the intervention groups (HFD-FA1 and HFD-M4A groups) was supplemented with (0.1 g/day) oligofructose (Spennrad, Germany) based on a water consumption of 4 mL/day per mouse. The purpose of oligofructose supplementation was to aid the administered bifidobacteria to survive and their adherence to mice intestinal mucus. The capability of bifidobacteria strains to adhere to the host intestinal mucosa is necessary for therapeutic manipulation and the adhesion has the capacity to be a strain dependent [17]. Bifidobacteria administration was started by the second week of the experiment. The administration was given in the light phase.

The food intake and the mice were both weighed weekly using a balance (Ohaus-Explorer Pro, Switzerland). The different groups of animals were fed as follows: 1, the HFD group was fed a high-fat diet; 2, the HFD-FA1 group was fed a high-fat diet and received *B. longum* subsp. *longum* FA1; 3, the HFD-M4A group was fed a high-fat diet and received *B. breve* M4A. At the end of the study, mice were euthanized under CO_2 with humane endpoints.

Daily Food Intake (FI) and mouse weight

FI, the amount of food ingested by the animals in each group was calculated. Mice were moved to clean cages and the amount of food given to them was weighed. Any food remaining in the previous cage was also weighed. These measurements were repeated weekly, and the FI (g/day per mouse) was calculated using the following equation (1) according to Ref. [18]:

$$\text{FI (g/day per mouse)} = (w_0 - w_{ed})/(n_d \times n_m) \ldots. (1)$$

The total energy intake was calculated as followed in equation (2):

$$\text{Total energy intake (kcal/mouse)} = (w_0 \times e)/(n_d/n_m) \ldots (2)$$

w_0=Weight (g) of the food provided, w_{ed}=Weight (g) of the food remaining in the cage at the end of the feeding period, n_d=number of days over which FI was calculated, *e*=amount of the energy in the diet, and n_m=number of mice per cage.

Serum triglycerides and cholesterol

Serum samples were stored at -80°C until use. The total serum cholesterol and triglyceride levels were measured using a Reflotron analyzer (Roche, Basel, Switzerland) following the manufacturer's instructions.

Diet composition (g/kg)	Control diet (10% kcal from fat)	High fat diet (70% kcal from fat)
Crude Protein (Casein)	210.89	210.36
Crude Fat*	38.80	436.14
Crude Fiber (Cellulose)	40.26	33.15
Crude Ash	46.96	48.96
Monosaccharide	26.24	68.95
Disaccharide	130.56	66.24
Polysaccharide (Starch)	413.40	102.45
Choline chloride	1.01	1.00
Vitamin premix**	0.563	0.551
Minerals premix**	36.01	31.32
Moisture	80.36	30.09
Energy (kcal/kg)	3,493.98	5,613.25

Table 1: Composition of experimental diets.
*Butter, lard and unsaturated fatty acids fat (Oleic acid and Linoleic acid); **According to the standard composition for mice feed prepared by Altromin-Germany; http://www.altromin.de

Bacterial count of cecal matter

The cecum and a portion of the adjacent colon tissue with content of each mouse were removed and placed in capped sterile tubes. After transfer to a laminar flow cabinet, 1 gram of each sample contents was transferred to a tube with 9 mL of Ringer (Sigma-Aldrich) solution (0.25 power) and homogenized by vortexing for 1 minute. Eight to nine-fold serial dilutions of each sample were performed, which were plated with selective mupirocin 100 mg/L MRS agar medium supplemented 0.5 (g/L) L-Cystein for Bifidobacteria count and the MRS agar medium was used for lactic acid bacteria count. Both media were incubated anaerobically at 37°C for 72 h in triplicate. The numbers of colony forming units (CFU) were expressed as log CFU/g.

Histology of liver tissues

Tissues were analysed histologically to assess the development of obesity and the morphological structures of the fatty liver. Liver tissues were fixed in paraformaldehyde, dehydrated in ethanol, cleared using xylene and then embedded in paraffin. The tissues in paraffin blocks were then sectioned and stained using haematoxylin (Roth, Germany) and eosin (Thermo Electron Corporation, USA).

Haematoxylin and eosin staining

Tissue slides were rehydrated with water then stained using haematoxylin and eosin which stained the nuclei dark blue and cytoplasm pink, respectively. Thise allows cell types to be differentiated and fat content, and cell size to be determined.

Oil red O staining

Cryoembedding of tissues Liver tissues sized 5 × 5 × 5 mm were harvested from frozen cryoembedded tissues and then frosted in cryomedium in embedding cryocassettes on dry ice. The frosted samples were stored at -80°C until analysis. The remaining liver samples were snap-frozen in liquid nitrogen.

Oil Red O-staining was used to detect lipid accumulation in liver tissues. To produce the stock solution, 0.5 g of Oil Red O was dissolved in 100 mL isopropanol with gentle heating in a water bath. Fresh stain was prepared for each experiment. Ten-micrometre sections were cut and then air-dried. Slides were fixed in 10% formalin or 4% paraformaldehyde, and washed briefly with running tap water for 1-10 min. They were then rinsed with 60% isopropanol and stained with freshly prepared Oil Red O working solution for 15 min. After rinsing briefly with 60% isopropanol, the nuclei were stained lightly with alum haematoxylin. Additionally, the slides wer rinsed with distilled water, and mounted in Dako® fluorescent mounting medium (Dako, USA) and covered with coverslips [19]. The staining of the lipid droplets were visualized using a light microscopy (LEITZ DMRB-Leica, Germany) at 200x magnification. Picture were taken with a digital camera (MXA 5400-Nikon). Lipid droplets size in microscopy images were determined by Image J program (2 × 2.1.7.4). The photos analyzed with binary settings and the area calculated. Dirty or air bubbled photos were avoided.

Statistical Analysis

The analyses were performed in triplicate. Statistical analyses were performed using SPSS version 20 (IBM software, USA). Comparison of the bacterial number was made after logarithmic transformation. Analyses of variance (ANOVA) was used for single comparisons (each treated group to the HFD group), and $p<0.05$ and $p<0.01$ were considered statistically significant. The post hoc LSD test was chosen for homogenous variances.

Results

Food intake

The total food consumption of seven weeks was 266.9 ± 4.33, 198.4 ± 55.26 and 165.1 ± 45.92 g per mouse in the HFD, HFD-FA1 and HFD-M4A groups, respectively (Figure 1a). The consumed diet of HFD-FA1 and HFD-M4A groups was decreased significant ($p<0.01$) in week 3 and decreased significant ($p<0.01$) of HFD-M4A group in week 4 in comparison with HFD group. The increase in total food intake in the HFD group was not significantly higher than the HFD-FA1 and HFD-M4A groups. Nevertheless, the increase in total food intake in the HFD group was not significantly higher than the HFD-FA1 and HFD-M4A groups. Regarding the Figure 1b, no significant difference was observed in total energy intake means 1498.55 ± 24.31, 1114.04 ± 310.2 and 926.81 ± 257.8 (kcal per mouse) the studied groups.

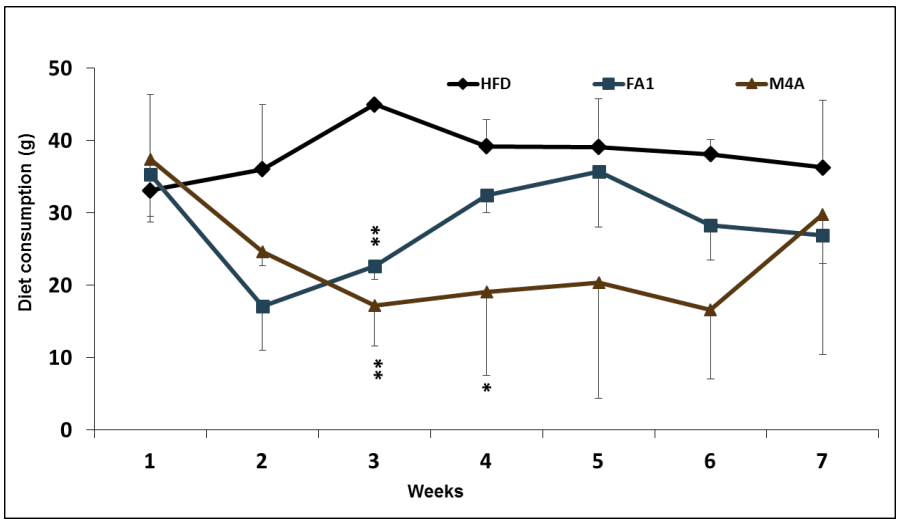

Figure 1a: Total diet consumption of mice treatments after six weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. The result is presented as the mean with standard error (mean ± SE; n=7); *p<0.05, **p<0.01 (vs. HFD group).

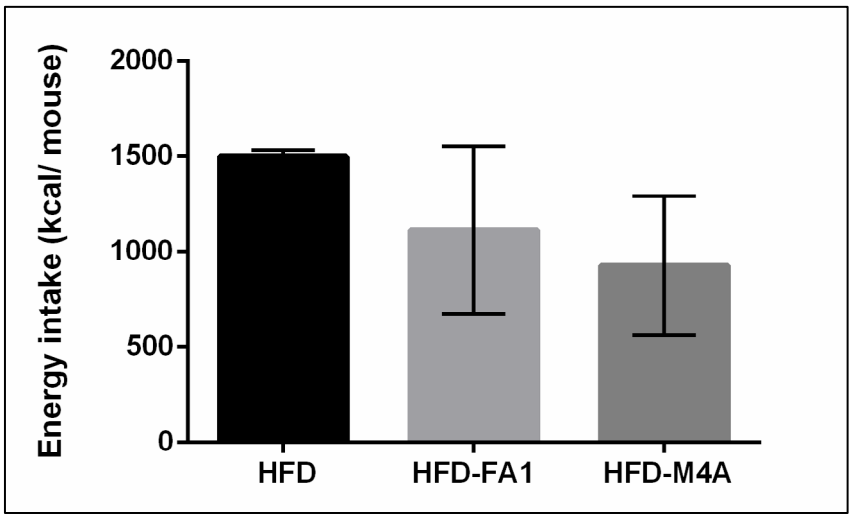

Figure 1b: Total energy intake in all groups at the last weekend of the experiment. The caloric density intake based on 5,613.25 kcal / kg. Data are expressed as mean ± SD (n=7).

Body weight and weight gain

A significant difference (p<0.01) in body weight was already observed after the start of the supplementation phase between (23.64 ± 0.69) HFD-M4A group and (26.34±0.59) HFD group, with a decreased weight in (22.25 ± 0.63) HFD-M4A group, however, Figure 2a showed body weight within seven weeks of the study. The administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 decreased weight gain significantly (p<0.01) in the HFD-M4A and HFD-FA1 groups compared with the HFD group that was 2.09 ± 0.84, 3.44 ± 0.53 and 5.54 ± 0.22 g per mouse, respectively (Figure 2b).

Bacterial counts in cecal content

Lactic acid bacteria: The numbers of lactic acid bacteria in cecal matter of the HFD-FA1 and HFD-M4A groups were 9.52 ± 0.015 and 9.62 ± 0.038 log CFU/g, respectively, which was increased significantly (p<0.01) in comparison with (6.68 ± 0.24 log CFU/g) the HFD group (Figure 3).

Bifidobacteria: The number of bifidobacteria in cecal matter increased significantly (p<0.01) in the HFD-M4A and HFD-FA1 groups was 8.80 ± 0.99 and 8.76 ± 0.30 log CFU/g, respectively, compared with 1.58 ± 1.37 log CFU/g in the HFD group (Figure 3).

Serum triglycerides: Figure 4 shows that the administration of *B. breve* M4A in the HFD-M4A group decreased triglyceride levels significantly (p<0.05) to 125.38 ± 15.01 (mg/dL) compared with 174.43 ± 13.27 (mg/dL) in the HFD-group. There were no significant differences in triglycerides between (160.33 ± 5.68 mg/dL) the HFD-FA1 and HFD groups. Total serum cholesterol level was not significantly different among groups.

Bifidobacteria and hepatic histology: The Oil Red O-stained area was calculated to assess triglyceride accumulation in the liver using Image J software. The proportion of the lipid droplets area (Figure 5a) in cryosection images was 12.43 ± 1.55% in the HFD group, which was significantly high (p<0.01) compared with 3.401.68% and 2.46 ± 1.84% in the HFD-FA1 and HFD-M4A groups, respectively.

Haematoxylin and eosin staining liver: Figure 5b shows images of haematoxylin and eosin-stained liver sections (100X and 1000X magnification). The haematoxylin and eosin-stained sections of the treatment groups showed cells we're being sized with less fat content (Figure 5b).

Discussion

Bifidobacteria are used as probiotics for the prevention and treatment of pathologies typical of newborns, such as necrotizing enterocolitis and streptococcal infections [20]. Furthermore, allergic diseases, celiac disease, obesity, and neurologic diseases potentially presented new opportunities for probiotic applications of bifidobacteria [20]. Short chain fatty acids are the primary end products of carbohydrate metabolism in bifidobacteria lowering the pH and reducing the growth of undesirable microorganisms in the intestine, as well as to increase peristalsis pushing the gut contents forward and eventually helping to remove pathogens [21]. Due to acetate production, *B. longum* subsp. *longum* strain JCM 1217T prevented the apoptosis of intestinal epithelial cells when *E. coli* O157 were administrated to germ-free mice. In previous work, we found high differences between to two bifidobacteria strains used regarding to the acid production in all tested media supplemented with different concentration of yeast extract, glucose and oligofructose, as compared with the control [13]. In the present study, two strains of bifidobacteria were used because of their origin from the human microbiota in breastfed infants. The reduction of weight gain and serum triglyceride commensurate with the amount of acid product, since several observations have been reported with *B. longum* subsp. *longum* FA1and *B. breve* M4A were studied [9]. Bifidobacteria isolates were chosen for higher viability as recommended for probiotic microorganisms [22].

Food consumption

Food consumption was recorded throughout the seven-week study period. Mice fed a high-fat diet and treated with *B. breve* M4A *B. longum* subsp. *longum* FA1 consumed a non-significantly different amount of food than other groups. Administration of *B. breve* M4A reduced total body weight gain compared to HFD only. Casas and Dobrogosz [23] revealed that *Lactobacillus reuteri* improved the intestine's ability to absorb and process nutrients and increase food conversion. Another study reported that *Lactobacillus reuteri* was the only species present at

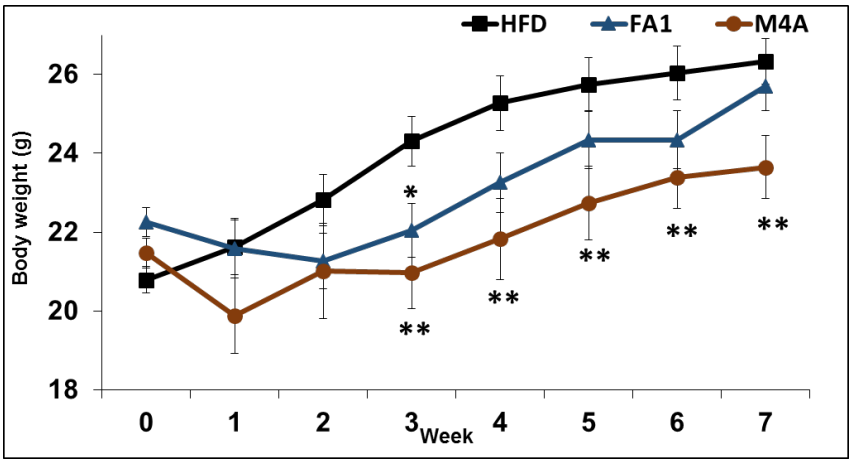

Figure 2a: Body weight development of the three treatment groups. One group received high-fat diet only, one received HFD and *B. breve* M4A (4.1×10^6 CFU/day), one received *B. longum* subsp. *longum* FA1 (2.9×10^6 CFU/day). From time 0 to 1, all groups were on control diet. HFD started from time point 1. Additional supplementation started at time point 2 and had a duration of six weeks. Data are expressed as mean ± SE (n=7); *Significant at ($p<0.05$); **Significant at ($p<0.01$).

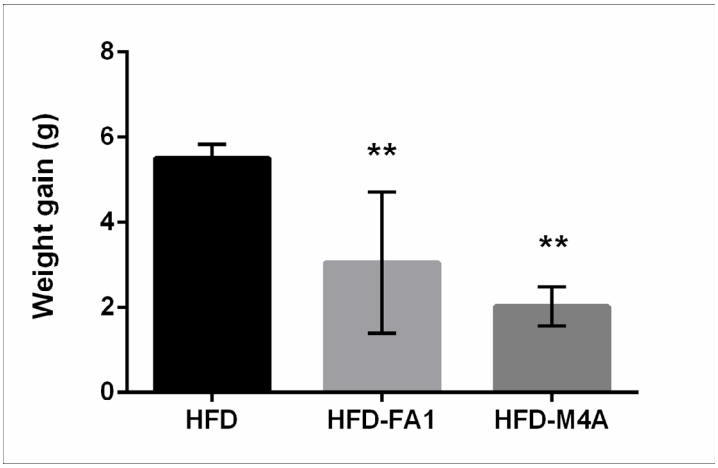

Figure 2b: Weight gain of mice after six weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1, for HFD-M4A group and HFD-FA1 group respectively. (mean ± SD, n=7); ** Significant at ($p<0.01$).

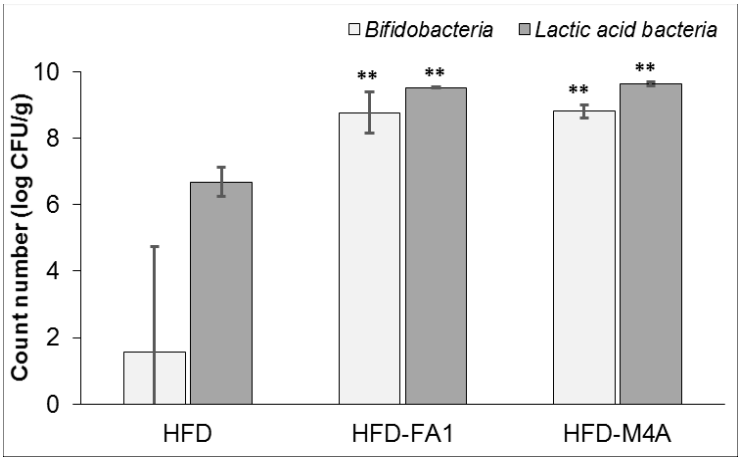

Figure 3: Count number of lactic acid bacteria and Bifidobacteria from cecal matter of mice treatments after administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1, for HFD-M4A group and HFD-FA1 group, respectively. (mean ± SD, n=7); ** Significant at ($p<0.01$).

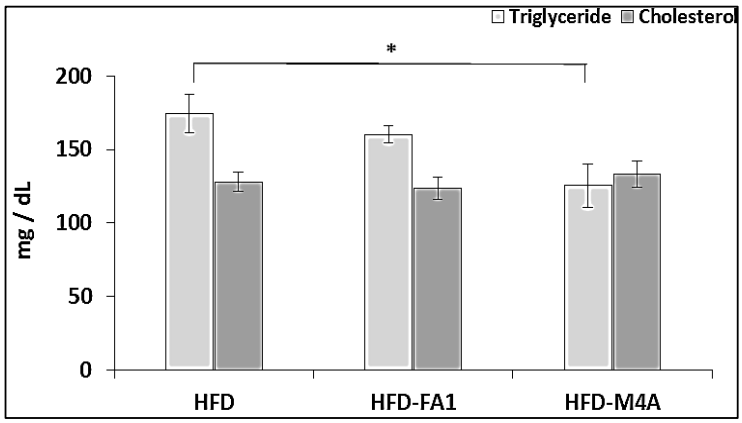

Figure 4: Total cholesterol and triglyceride levels in serum of mice after six weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. (mean ± SE, n=7); *Significant at ($p<0.05$).

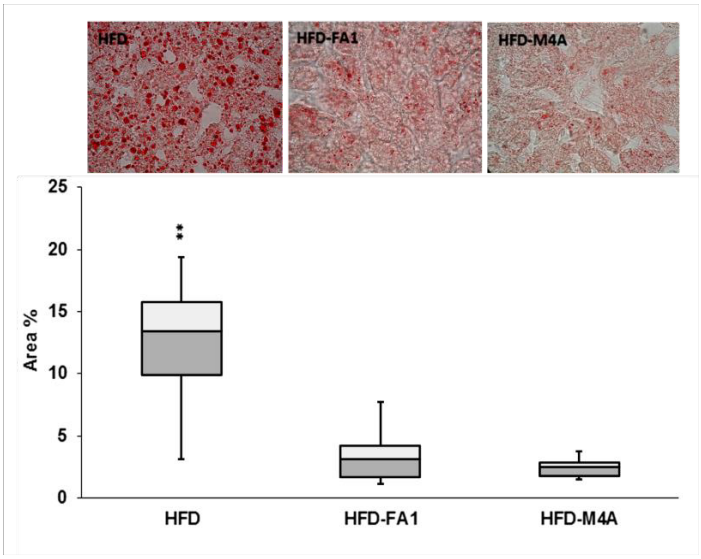

Figure 5a: Calculated area (%) of Oil Red O-staining photos imaging cryosections to determine triglyceride accumulation in liver by Image J program. HFD: high fat diet; HFD-FA1: high fat-diet+*B. longum* subsp. *longum* FA1; HFD-M4A: high-fat diet+*B. Breve* M4A. Photos A, B and C Were Oil Red O-stained native cryo-lices/ liver. Microvascular lipid droplets; altered by bifidobacteria. Magnif. 200X and 1000X, bars 200 µm (mean ± SE, n=7); ** Significant at ($p<0.01$)

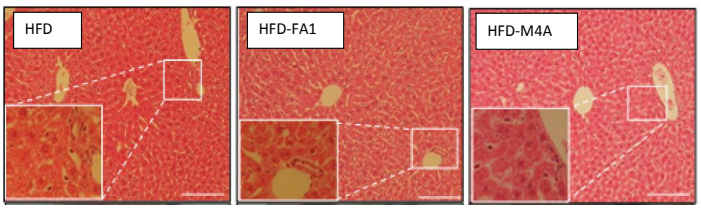

Figure 5b: Haematoxylin eosin-stained paraffin slices of liver showing microvascular lipid droplets. Magnification 100X and 1000X, bars 100 µm. HFD: high fat diet; HFD-FA1: high fat diet+*B. longum* subsp. *longum* FA1; HFD-M4A: high-fat diet+*B. Breve* M4A.

higher levels in the gut microbiota of obese individuals whereas levels of *B. animalis* and *Methanobrevibacter smithii* were higher in non-obese subjects [24]. A high-fat diet is considered one of the environmental factors that contributes most to the epidemic of obesity [25]. In the current study, mice fed a high-fat diet (HFD) had an increased body weight. The influence of six weeks of exposure to the HFD on weight gain was a positive fat balance. The gut microbiota produces energy from undigested food [26].

Body weight and weight gain

The administration of *B. breve* M4A might modulate the composition of the gut microbiota in the regulation of energy homeostasis. Another variable that might influence weight gain was observed in the HFD-FA1 and HFD-M4A groups. *B. breve* M4A and *B. longum* subsp. *longum* FA1 may lead to an altered intestinal microbiota that minimizes the amount of energy stored in adipose tissue,

thereby, reducing weight gain. The proposed mechanism was that gut microbiota play an important role in energy homeostasis in the host's intestine [26]. Thus, weight gain was less in these two intervention groups. *B. breve* M4A and *B. longum* subsp. *longum* FA1 might reshape the balance of the intestinal microbiota and modulate body weight.

Each *B. breve* strain may have a different capability of carbohydrate utilization as another study suggested. The genetic accessibility and murine colonization capacity of this strain makes it a valuable model for understanding bifidobacterial-host interactions in the gut [27]. Maximal counts and generation times of *B. breve*, *B. bifidum*, *B. infantis*, and *B. longum* were not influenced by 0.5% fructooligosaccharides (FOS) when the organisms were grown in infant formula (Dubey et al.), but the group did not investigate FOS-concentrations above 0.5%. Consistent with these findings, Shin [28] observed significant differences in mean doubling times when the bifidobacteria strain was grown in the presence of 0.5% FOS. Through the valuable content of amino acids, the yeast extract may contribute to the growth stimulation of bifidobacteria and also to improved viability in dairy products [29].

Serum triglycerides and total cholesterol

The mean triglyceride level (125.3 mg/dL) in the HFD-M4A group was reduced significantly in comparison to the HFD group. However, the triglyceride concentration in HFD-fed mice (174.4 mg/dL) was above the normal range (71-164 mg/dL) in adult mice [30]. The administration of *B. breve* M4A reduced plasma triglycerides to the normal range. This result is in line with a previous study which demonstrated lower serum and liver triglyceride levels in a murine obesity model after treatment with different *Bifidobacterium spp.* strains. *Bifidobacteria* M13-4 was associated with a significant increase in body weight while *Bifidobacteria breve* L66-5 resulted in a decrease in body weight based on similar energy consumption [12].

The high-fat diet used in the current study contained 44% crude fat with the same proportion of cholesterol as the control diet; therefore, there was no significant difference in serum cholesterol among the groups. In a previous study, the administration of 10^8 or 10^9 (CFU/day) *B. breve* B3 in six-week-old mice for eight weeks suppressed body weight gain and epididymal fat, and reduced serum cholesterol [16]. This is in line with our data even though it occurred under different conditions - e.g., age of mice, diet composition, and time of treatment.

Lactic acid bacteria and bifidobacteria

Mice fed a HFD exhibited an altered composition of the gut microbiota and different levels of lactic acid bacteria. The administration of *B. longum* subsp. *longum* FA1 and *B. breve* M4A increased the proportion of lactic acid bacteria and bifidobacteria. These species contribute to a beneficial intestinal microbiota [31]. The number of bifidobacteria was found inversely correlated with fat mass development by others already [23].

The dose of bacteria was chosen depending on the ability of bifidobacteria to grow in milk aiming at the number of viable cells required to meet the probiotic definition criteria. Mice in the intervention groups were given a daily oral dose (10^6 CFU) of viable cells in 0.2 mL, the maximum probiotic fermented milk volume that can be administered to mice [32]. Following the experience, the doses of bifidobacteria were increased in milk-supplemented media as much as possible. For achieving the beneficial effects of probiotics scientists have proposed that the product should contain probiotic bacteria with a minimum viable number of 10^6 CFU/mL and recommended 10^8 CFU/mL or per gram [32,33]. Various authors have reported a low viability of Bifidobacteria in fermented dairy foods [34]. The ability of organisms to grow well in milk depends on their ability to metabolize milk protein and lactose, and this ability varies considerably among strains [35].

Bifidobacteria reduced liver lipid droplets and liver weight

The main histological characteristic of non-alcoholic fatty liver diseases is the accumulation of fat in the form of triglycerides in liver cells [36]. The triglyceride content was measured in liver samples to investigate whether the bifidobacteria strains (*B. longum* subsp. *longum* FA1 and *B. breve* M4A) reduced hepatic fat accumulation. A reduction of lipid droptlet leads to a lower risk of metabolic diseases [37].

Oil Red O-stained and haematoxylin and eosin-stained

The accumulation of lipid droplets in Oil Red O-stained images of liver sections was decreased by the administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1. Haematoxylin and eosin-stained images of hepatic tissue from the HFD-fed mice group revealed a progressive increase in steatosis. Lee [38] evaluated the effects of fermented lotus extracts on inhibiting differentiation in 3T3-L1 pre-adipocytes. Different probiotics, including *L. plantarum*, *L. rhamnosus*, *B. breve*, and *B. longum,* were used separately. In their study the inhibition of pre-adipocyte development was examined using Oil red O dye staining and all groups fermented by four kinds of probiotics showed reduction in triglyceride deposition.

In obese animals and humans triglyceride storage in adipocytes is increased. The elevated number of lipid droplets in skeletal muscle and liver cells causes hyperlipidemia [37,38]. In the present study the hepatic mass of mice fed a high-fat diet was negatively correlated with the administration of Bifidobacteria. Thus, oral supplementation with *B. breve* M4A attenuates hepatic fat accumulation.

The quantitative analysis of liver triglycerides did not correlate to the histological outcome of lipid staining of the microscopic slides from liver samples. As the calculation of the sample size was based on weight gain and not on the liver triglyceride levels, a significant reduction may be observed with an higher sample sizes in future studies. Dhiman [39] assessed the efficacy of a probiotic preparation that could prevent the recurrence of hepatic encephalopathy, reduce the number of hospitalizations, and diminish the severity among cirrhosis patients. The intervention used in their study comprised daily intake of probiotics consisting of *L. acidophilus, L. paracasei, L. delbrueckii* subsp. *bulgaricus, L. plantarum, B. breve, B. infantis, B. longum* and *Streptococcus thermophiles* for six months. The reduction was significant in the development of hepatic encephalopathy among patients receiving the probiotic in comparison to in the placebo group. The Child-Turcotte-Pugh and model for end-stage liver disease scores were used to assess the prognosis of chronic liver disease, mainly in patients with cirrhosis. At 24 weeks of the study, there was clinically significant progress in parameters among the patients treated with the probiotic which modulated the gut microbiota by altering numbers, composition, and functions of bacteria, finally resulting in a reduction of serum ammonia levels.

The results of this study suggest that bifidobacteria supplementation may be helpful to reduce weight gain, to reduce serum triglyceride concentrations and to reduce hepatic steatosis under high fat diet. Future studies may examine effect of bifidobacteria supplementation on mice with established steatosis and obesity, but fed a low-energy diet.

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Statement of authors' contributions to the manuscript

The authors listed below have certified that they meet the criteria for authorship. They have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise. They take public responsibility for their part of the paper, except for the responsible author who accepts overall responsibility for the publication. All authors read and approved the final manuscript. Mustafa Alsharafani had responsibility for all parts of the manuscript.

- Mustafa Alsharafani and Michael Krawinkel planned and performed experiments, experimental procedures, specimen collection, experiment design, statistical analyses, responsible for animal treatment, analyzed the results, wrote the manuscript and primary responsibility for final content.
- Elke Roeb and Martin Roderfeld supported and aided pathological analysis and revised the drafted manuscript.

Compliance with ethics requirements

All co-authors declare no conflict of interest. This article does not contain any studies with human subjects.

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