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Intestinal removal of free fatty acids from hosts by *Lactobacilli* for the treatment of obesity

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Recent findings on the association of gut microbiota with various diseases, including obesity, prompted us to investigate the possibility of using a certain type of gut bacteria as a safe therapeutic for obesity. *Lactobacillus* mutants with enhanced capacity in absorption of free fatty acids (FFAs) were isolated to show reduced absorption of FFAs by the administered host, attributing to inhibition of body weight gain and body fat accumulation as well as amelioration of blood profiles. Consequently, high throughput screening of natural FFAs-absorbing intestinal microbes led to the isolation of *Lactobacillus reuteri* JBD30 l. The administration of *Lactobacillus* JBD30l lowered the concentration of FFAs in the gut fluid content of small intestine, thus reducing intestinal absorption of FFAs whereas promoting fecal excretion of FFAs. Animal data also confirmed that the efficacy of *Lactobacillus* JBD30l on body weight similar to that of orlistat, an FDA-approved pharmaceutical for long-term use to treat obesity. In a subsequent random, double-blind, placebo-controlled clinical trial (KCT0000452 at Clinical Research Information Service of Korea), there was a statistically significant difference in the percentage change in body weight between the *Lactobacillus* JBD301 and the placebo group ($P = 0.026$) as well as in the BMI ($P = 0.036$) from the 0-week assessment to the 12-week assessment. Our results show that FFA-absorbing *Lactobacillus* JBD301 effectively reduces dietary fat absorption, providing an ideal treatment for obesity with inherent safety.

Obesity is a disease of energy balance, characterized by a chronic disequilibrium between energy intake and expenditure [1]. It is associated with an increased risk of various chronic diseases, including hypertension, dyslipidemia, type 2 diabetes, cardiovascular disease, obstructive sleep apnea, osteoarthritis and cancer [2].

Abbreviations
BMI, body mass index; CFU, colony-forming unit; CNS, central nervous system; CT, computerized tomography; DEXA, dual-emission X-ray absorptiometry; DIO, diet-induced obesity; FFAs, free fatty acids; GI, gastrointestinal; HDL, high-density lipoprotein cholesterol; HFD, high-fat diet; LDL, low-density lipoprotein cholesterol; MRI, magnetic resonance imaging; MRS, Man–Rogosa–Sharpe; NTG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; SCFA, short-chain fatty acids; SD, Sprague–Dawley; TC, total cholesterol; TG, triacylglycerol; USFA, unsaturated fatty acids.

Because obesity is rarely curable and its prevalence has increased over several decades, intensive research has been conducted to develop anti-obesity drugs, resulting in many candidates with very interesting anti-obesity effects at preclinical levels [3–5]. The path to drug development for obesity, however, has been littered with failures in clinical development and withdrawals from the market due to severe side effects [6–8]. Orlistat, the major FDA-approved pharmaceutical for long-term use to treat obesity, is a gastric and pancreatic lipase inhibitor that prevents fat hydrolysis, thus reducing dietary fat absorption by ~ 30% [9–11]. Despite substantial anti-obesity effects, the inhibition of lipase activity by orlistat generates undigested fat in the gastrointestinal (GI) tract, which causes side effects that are not only uncomfortable but also socially unacceptable [12].

Unlike orlistat, several appetite suppression drugs were successfully approved and marketed for obesity treatment, but these drugs have been withdrawn due to severe psychiatric and/or cardiovascular side effects, all common adverse effects of central nervous system (CNS)-acting drugs [13]. The FDA recently approved two new anti-obesity drugs that work on the CNS, lorcaserin and phentermine/topiramate, driven by high demand for anti-obesity drugs [14–16]; however, the future of these new drugs remains uncertain, considering the history of anti-obesity drugs that work on the CNS. Because of the safety issues with anti-obesity drugs that work on the CNS [17], anti-obesity drugs with different modes of action are under investigation [18,19]. Drugs targeting pathways in the metabolic tissues, the peptidergic signaling systems of hunger and satiety, and the homeostatic mechanisms related to leptin have shown potential in preclinical studies, but none of the drugs has been safe and effective in clinical development thus far. Therefore, a new type of anti-obesity treatment must be actively sought because the current pharmaceutical drugs are not ideal for the treatment of obesity.

A new paradigm is obviously necessary to develop anti-obesity drugs engendering sustained weight loss with minimal side effects. Recent evidence showed that the gut microbiota play an intricate role in the regulation of body weight [20–22]. Transplantation experiments using microbiotas from either obese or lean mice and from either obese or lean humans into germ-free mice proved that the compositional change in microbiota in the GI tract resulted in differences in the efficiency of caloric extraction from food, eventually contributing to different body weights [21,22]. These results suggest that small changes in caloric extraction from the GI tract by transplanting a specific intestinal bacterium can lead to a meaningful reduction in body weight. In fact, attempts have been made to identify specific intestinal bacterium to control obesity. Interestingly, a few probiotic strains have been shown to ameliorate obesity and metabolic disorders, without clear understanding of underlying mechanisms [23,24].

Dietary fat is the major contributor in our caloric extraction from food. Because fat is degraded into FFAs before absorption into the body, the removal of FFAs in the GI tract by the transplantation of a FFAs-absorbing bacterium might be an ideal choice for treating obesity by decreasing fat uptake by the host body. Transplanted microbes with enhanced capacity in FFA absorption would compete for FFAs with enterocytes in the intestinal epithelium, resulting reduced FFA absorption and thus lowered caloric intake into host. In fact, recent study have shown that microbiota contribute obesity by stimulating intestinal FFA absorption in the zebrafish model, suggesting inhibition of intestinal FFA absorption and lipid droplet formation to regulate host obesity [25]. Considering that increased FFA along with hyperglycemia are the key hallmarks of obesity, metabolic syndrome and diabetes, FFA provides an excellent metabolic target to reduce dietary energy harvest and thus prevent and counteract obesity. In addition, reduction in caloric extraction with FFAs-absorbing bacteria may be a better choice than inhibiting fat hydrolysis by orlistat, which results in an unavoidable problem with undigested fat.

Based on the fact that orlistat, which inhibits digestion of dietary fat to FFAs, is currently the best anti-obesity drug, as well as the fact that small changes in caloric extraction affected by intestinal microbiota could lead to large body weight differences, we investigated whether the inhibition of FFAs absorption into the human body by using an intestinal bacterium would lead to the development of anti-obesity drugs producing sustained weight loss without side effects. As expected, the administration of FFAs-absorbing lactobacilli that remove intestinal FFAs before absorption to host showed significant anti-obesity effects, with efficacy as high as orlistat in animal experiments and a clinical trial. Our results not only provide a novel Lactobacillus approach as a safe way to prevent or treat obesity but also suggest the feasibility of developing treatments by modulating the metabolic activities of the intestinal microbes.

Materials and methods

Materials

Reagents and kits were purchased from Sigma, except for the following: [1-^{14}C]-palmitic acid (PerkinElmer Life

Sciences, Santa Clara, CA, USA), liquid scintillation cocktail (LSC, PerkinElmer Life Sciences), [carboxyl-^{14}C]-triolein (PerkinElmer Life Sciences), Man–Rogosa–Sharpe (MRS, Becton Dickinson, NJ, USA), orlistat (Xenical, Roche, Basel, Switzerland), EnzyChrom™ Free Fatty Acid Assay Kit (BioAssay Systems, Hayward, CA, USA). The sterilizable 384-well plate and 384-pin replicator were from Nunc. The membrane semidry system was from Bio-Rad, and the X-ray film was from Kodak. The GEL-PRO ANALYZER software was from Media cybernetics. The magnetic resonance imaging (MRI) images were obtained with a Bruker Biospec 47/40 4.7-Tesla instrument (Bruker, Billerica, MA, USA) and analyzed with IMAGE J software (NIH, Bethesda, MD, USA). The serum was analyzed with a Rat/Mouse ELISA kit (LINCO research, St. Charles, MO, USA), a Leptin ELISA kit (R&D System, Minneapolis, MN, USA), a blood glucose meter (Accu-Chek, Roche) and Cholesterol ELISA kits (Asan Pharmaceutical, Seoul, Korea).

Isolation of FFAs-absorbing *Lactobacillus* mutants

Lactobacillus acidophilus KCTC 3179 is a human-derived *Lactobacillus* strain from the Korea Collection for Type Cultures (KCTC, Daejeon, Korea). Anaerobic culture was carried out in an anaerobic jar (BBL Gas-Pack anaerobic systems, Apeldoorn, Netherland). *Lactobacillus acidophilus* KCTC 3179 were chemically mutagenized by using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) to select the *Lactobacillus* mut1 followed by 2nd-round mutagenesis by using 4-nitroquinoline-1-oxide to select *Lactobacillus* mut2 that absorbs FFAs strongly. FFAs-absorption ability of the mutants was evaluated *in vitro* by measuring radioactivity with liquid scintillation spectrophotometry after incubation for 30 min with ^{14}C-labeled palmitic acid. For *in vivo* evaluation, Sprague–Dawley (SD) rats had been fed standard diet (60% complex carbohydrates, 22% protein, 3.5% fat, 5% fiber, 8% crude ash, 0.6% calcium and 1.2% phosphorus) supplemented with testing mutants at 10^7 CFU·day^{-1} for 8 weeks. Intestinal colonization of mutants was confirmed by counting. Then, blood at 2 h, 4 h, 6 h, 8 h and 10 h were analyzed for radioactivity after feeding ^{14}C-labeled triolein whereas FFAs were analyzed with fecal fluid content with GC/MS.

Animal experiments

All animal care and use were performed strictly in accordance with the ethical guidelines by the Ethics Committee of Chonbuk National University Laboratory Animal Center and the animal study protocol was approved by the institution (CBU No. 2012-0040).

Anti-obesity effects of FFAs-absorbing *Lactobacillus* mutants

The anti-obesity effects were assessed under diet-induced obesity (DIO) condition with SD rats fed high-fat diet (HFD, 48% complex carbohydrates, 17.6% protein, 22.8% fat, 4% fiber, 6.4% crude ash, 0.48% calcium, and 0.96% phosphorus) supplemented with testing mutants at 10^7 CFU·day^{-1} for 22 weeks. The body weights of the SD rats were measured weekly. At the end of the experiments, MRI analysis was performed to measure the visceral (or abdominal) fat with a Bruker Biospec 47/40 4.7-Tesla instrument. Blood samples from the experimental rats were collected with overnight fasting at 0 and 22 weeks. The sera were analyzed for biochemical characteristics using commercially available kits. The serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) levels as well as the triacylglycerol (TG) concentrations were detected with the ELISA kits described in the Reagents section above.

Identification of intestinal FFAs-absorbing *Lactobacillus* JBD301

Lactobacillus strains were isolated from the feces of healthy lean adult volunteers. For individual lactobacilli overnight cultures, the quantities of FFAs in the cultured media were determined using the EnzyChrom™ free fatty acid assay kit (Bio-Assay Systems, Hayward, CA, USA) as described by the manufacturer. Using the concentration of FFAs in the media, the quantity of FFAs uptake from the media was calculated. A natural *Lactobacillus* strain with the strongest FFAs-absorbing ability was identified, taxonomically classified and phylogenetically analyzed by 16S rDNA sequences (NCBI GenBank, DNA data bank of Japan, European Nucleotide Archive, www.phylogeny.fr).

Animal studies of *Lactobacillus* JBD301

Seven-week-old female C57BL/6 mice were fed HFD supplemented daily with 10^7 CFU of *Lactobacillus* JBD301 for 3 weeks. Then, gut fluid contents were collected and analyzed for total FFAs concentration at small intestine and large intestine of the mice with the EnzyChrom™ FFAs assay kit. To evaluate changes in FFAs absorption and excretion by host fed *Lactobacillus* JBD301, the radioactivities of the bloods as well as feces were measured after feeding 1 μCi of ^{14}C-labeled triolein.

Clinical trial of *Lactobacillus* JBD301

A phase 2, double-blind, placebo-controlled human study was conducted with *Lactobacillus* JBD301 at the Samsung Medical Center (Seoul, Korea). The research protocol was

approved by the Clinical Trial Center at the Samsung Medical Center and the methods were carried out in accordance with the approved guidelines (SMC No. 2011-04-061-002). This trial was registered with Clinical Research Information Service of Korea as KCT0000452 (https://cris.nih.go.kr). The purpose of this study was to evaluate the safety and tolerability of *Lactobacillus* JBD301 and to evaluate the efficacy of *Lactobacillus* JBD301 compared to placebo for the reduction in body fat or weight in adults with $25 \leq \text{BMI} < 35$. Among the recruited subjects, 37 subjects were randomly assigned to either the placebo group or experimental group. Placebo group ($n = 19$) were administered single capsule of vegetable cream as control whereas experimental group ($n = 18$) were administered *Lactobacillus* JBD301 at dose of 1×10^9 CFU·day^{-1} for 12 weeks. For the clinical data, the statistical analysis was performed using procedures in SAS (Version 9.2) and MEDCALC (Version 11.6.0). Depending on the normality of the underlying data, the Mann–Whitney *U* test and the Wilcoxon signed rank test were used to perform statistical analysis.

Further details of the materials and methods used in this study can be found in the supplementary material.

Results

Lactobacillus mutants with enhanced FFAs absorption reduced FFAs absorption by host

To test the hypothesis that obesity can be controlled by an intestinal bacterium that removes obesity-causative metabolites, i.e. FFAs, in the gut, a commercial probiotic strain, *L. acidophilus* KCTC 3179, was mutagenized by NTG to isolate mutants that strongly absorb palmitates, the most common form of FFAs. We initially isolated a mutant that absorbed 2.1 times more ^{14}C-palmitate from its surrounding environment than the parental strain (Fig. 1A). The identified mutant, mut1, was further mutagenized by 4-nitroquinoline 1-oxide, resulting in mut2, which absorbed FFAs 3.2 times more strongly than the parental strain (Fig. 1A). For the identified mutants, the acidification abilities during growth and colonization in the host GI tract after consumption were examined because these are the most important characteristics of edible *Lactobacillus* [26]. Both mutant strains showed normal growth and acidification activities during milk fermentation (Table S1). They also successfully colonized the GI tract of rats (Table S2). These results indicate that both mutants function as normal lactobacilli, except for their stronger absorption of FFAs.

When consuming lactobacilli, the bacteria transiently colonize the small intestine [27], where most of FFAs are absorbed into the body. Therefore, the administered mutants could actively remove intestinal FFAs by functioning as a bio-sequestrant, reducing the amount of FFAs available to be absorbed into the host body and thereby reducing dietary fat absorption. To test whether the mutants actually reduce the amount of absorbable FFAs in the GI tract of the host, SD rats were fed *Lactobacillus* for daily administration of 10^7 CFU for 8 weeks. After colonization, rat feed containing radiolabeled dietary fat, ^{14}C-triolein, was orally administered to the rats so that the amount of FFAs absorbed from dietary fat could be measured by measuring the radioactivity of the FFAs in their blood (Fig. 1B). Compared to the high-fat diet (HFD) control, the rats colonized with the both mutant strains showed a significant decrease in the amount of absorbed FFAs, whereas those colonized with parental strain 3179 showed no significant change. At 240 min after administration of radiolabeled dietary fat, the radioactivity of absorbed FFAs in the blood of the rat colonized with mut1 and mut2 were down to 298.4 cpm and 206.6 cpm, whereas control and parental strain 3179 group were 546.9 and 446.6 cpm respectively. As shown in Fig. 1B, the rats colonized with mut1 and mut2, but not parental strain, absorbed significantly less FFAs than the uncolonized controls.

If those mutants absorb FFAs strongly in the GI tract and thus remove FFAs available for the GI tract of the host, the FFAs quantities in the feces should decrease in the rats colonized with the mutants. We performed GC/MS analysis on the fecal fluid contents from *Lactobacillus*-fed rat, as described previously [28]. As expected, the total FFAs quantities in the fecal fluid content were significantly lowered in the rats colonized with the mutants compared to the uncolonized control whereas parental strain show some reduction (Fig. 1C). Most dramatic changes were with saturated fatty acids, particularly, stearic acid and palmitic acid. Unsaturated fatty acids (USFA), such as oleic acid and arachidonic acid were also reduced as well as short-chain fatty acids (SCFA), such as acetic acid, propanoic acid, butanoic acid, valeic acid, pentanoic acid and hexanoic acid. Particularly, pentadecanoic acid, palmitelaidic acid, nonadecanoic acid, heptanoic acid, heneicosanoic acid, sebacic acid, dodecanoic acid were reduced to undetectable ranges in the mut2 group. This overall decrease in FFAs including short-chain FA in the liquid portion of the fecal matter indicates that the mutants actively absorbed and removed intestinal FFAs in the colonized GI tract as shown in fecal fluid, thereby reducing the amount of FFAs available to be absorbed by the host.

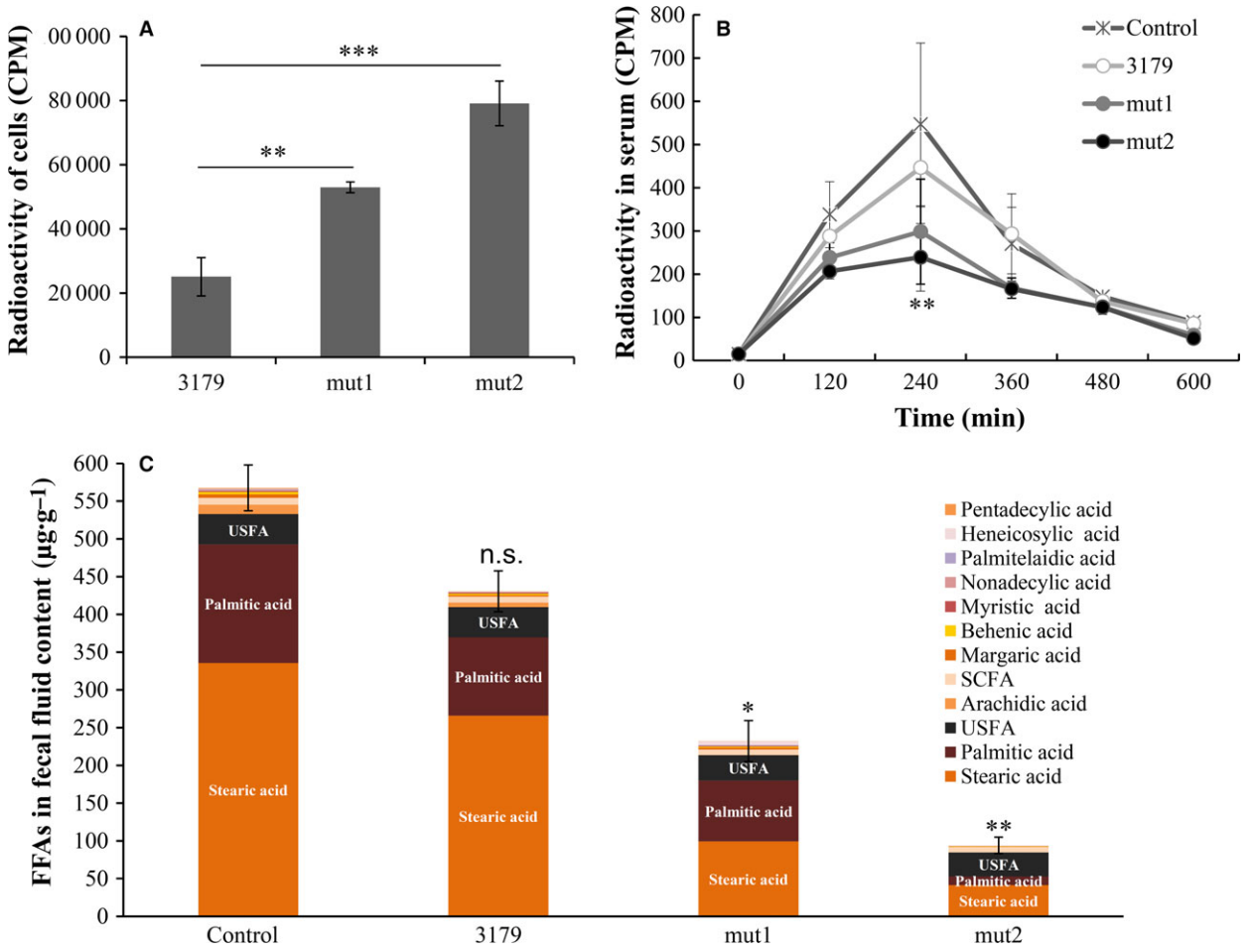

Fig. 1. Administration of the FFAs-absorbing *Lactobacillus* mutants significantly reduced the absorption of FFAs by the host. (A) The FFAs absorption by lactobacilli (*L. acidophilus* KCTC 3179, mut1 or mut2) was determined by measuring the radioactivity in lactobacilli after incubation with ^{14}C-labeled palmitic acid for 30 min. Values are mean ± SD (n = 4). (B) Three-month-old male SD rats were randomized and fed a standard diet only (control) or a diet-supplemented daily with 10^7 CFU of tested lactobacilli (*L. acidophilus* KCTC 3179, mut1 or mut2). After 8 weeks, rat feed containing radiolabeled fat, ^{14}C-triolein, was administered. Blood samples were collected at the indicated times and radioactivity was analyzed to quantify the amount of FFAs from absorbed dietary fat in the blood of hosts. Values are mean ± SD (n = 9). (C) FFAs in the fecal fluid contents from the hosts were analyzed by GC/MS. Major saturated fatty acids, stearic acid and palmitic acid, were indicated in the bar. Unsaturated Fatty Acid (USFA) includes oleic acid, arachidonic acid. Short-chain Fatty Acid (SCFA) includes acetic acid, propanoic acid, butanoic acid, valeric acid, pentanoic acid, hexanoic acid. Values are mean ± SEM (n = 4). Statistical significance is shown as *P < 0.05, **P < 0.01 and ***P < 0.001 versus control by ANOVA.

FFAs-absorbing *Lactobacillus* mutants ameliorated obesity in rats

After confirming the ability of the mutants to remove FFAs in the GI tract, the anti-obesity effect of the FFAs-absorbing mutants was evaluated by feeding the mutant to rats under diet-induced obesity (DIO) conditions (Fig. 2A). Daily administration of parental strain 3179 or its mutants resulted in successful colonization in the GI tract of the rats after 4 weeks (Table S3). Daily administration of mut1 and mut2 for 22 weeks resulted in consistent reduction in body weight gain (Fig. 2A). At the end, the body weights of uncolonized control, parental 3179, mut1 and mut2 were 514 g (100%), 507 g (101.4%), 435 g (85%) and 424 g (82%), respectively, with maximum difference of 15% for mut1 and 18% for mut2, respectively, compared to the uncolonized control.

In addition to body weight, visceral fat is correlated with obesity as the intake of excess calories in mammals primarily accumulates as visceral fat [29,30]. The visceral fat areas from untreated control, parental strain, mut1 and mut2 were measured 27%, 24%, 14% and 13%, respectively, at 22 weeks using an

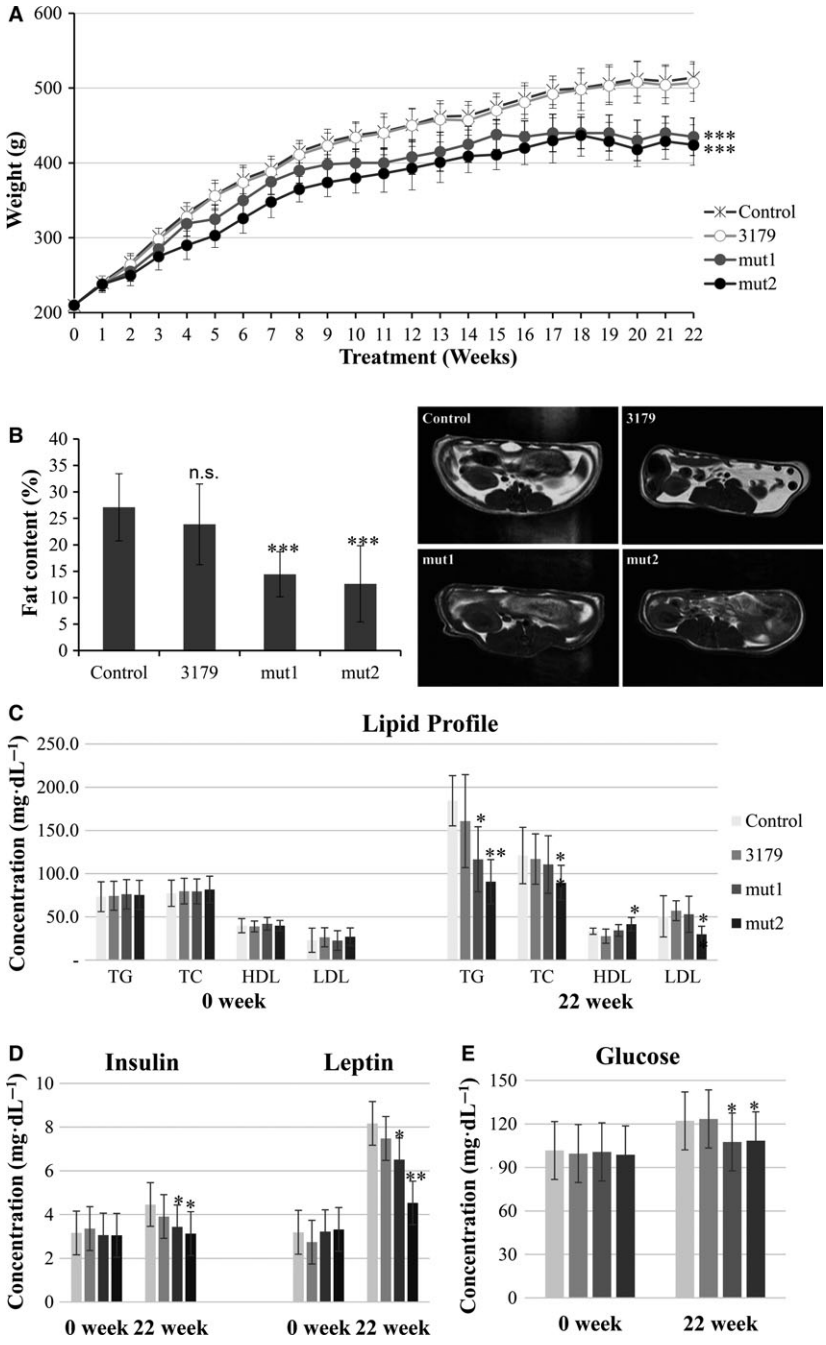

Fig. 2. FFAs-absorbing *Lactobacillus* mutants effectively inhibited weight gain and body fat accumulation and also ameliorated blood profiles under diet-induced obesity condition. Three-month-old male SD rats were randomized and fed a HFD only (control) or a HFD diet supplemented daily for 22 weeks with 10^7 CFU of tested lactobacilli (*L. acidophilus* KCTC 3179, mut1, or mut2). (A) Body weights were monitored weekly. (B) The change in visceral fat areas of the rats. The representative MRI images of visceral fat accumulation were shown. (C) The change in plasma lipid profiles. TG, triacylglycerol; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol. (D) The change in plasma insulin and leptin concentrations. (E) The change in blood glucose concentrations. Values are mean ± SD ($n = 14$). Statistical significance is shown as $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control by ANOVA.

open-type 0.3 Tesla MRI (Fig. 2B). These results indicated that the administration of the FFAs-absorbing *Lactobacillus* mutants could reduce FFAs absorption by the host and thus reduce body weight as well as visceral fat accumulation.

As the body gains weight, it becomes less sensitive to insulin and leptin, which leads to increased plasma concentrations of leptin, insulin and glucose as well as increased LDL and TC [31,32]. The blood profile with respect to the obesity markers was also analyzed at the beginning and end of the feeding experiments. There were significant differences in the lipid profiles within the experimental groups whereas parental 3179 strain failed to show significant differences (Fig. 2C). TG levels of mut1 and mut2 were 63% and 49% of the control respectively. In TC, HDL and LDL, however, only mut2 showed significant difference compared to the uncolonized control. TC levels of mut1 and mut2 were 91% and 74% of the control whereas LDL levels were 104.5% and 58.8% of the control respectively.

HDL levels of mut1 and mut2 were 103.1 and 124.9% of the control respectively.

Feeding the rat mut1 and mut2 also significantly reduced the serum insulin levels by 23% and 30%, and serum leptin levels by 30% and 45%, respectively, compared to the uncolonized control (Fig. 2D). The mutant strains also exhibited glucose-lowering effect, which was expected from their anti-obesity effect (Fig. 2E). The serum glucose levels were lower in the mut1 and mut2 groups (107.6 mg·dL^{-1} and 108.4 mg·dL^{-1}, respectively) than in the uncolonized control and parental strain groups (122.1 mg·dL^{-1} and 123.4 mg·dL^{-1}, respectively). Taken together, our FFAs-absorbing lactobacilli not only inhibited weight gain and body fat accumulation but also improved blood profiles, ameliorating obesity.

Isolation and characterization of intestinal FFAs-absorbing *Lactobacillus* JBD301

Because the mutant experiments proved our hypothesis that energy intake can be reduced with the FFAs-absorbing *Lactobacillus*, we performed high throughput screening to identify intestinal FFAs-absorbing *Lactobacillus* strains. Fecal samples from lean volunteers were inoculated on MRS agar plates, a selective media for *Lactobacillus*. By screening more than 20 000 strains, we were able to find various *Lactobacillus* strains that absorbed FFAs stronger than the common *Lactobacillus* strains in a FFAs quantitative assay. Animal experiments using the identified FFAs-absorbing *Lactobacillus* strains in C57BL/6 mice showed that the degree of efficacy on weight gain was positively correlated with the FFAs absorption degree of the *Lactobacillus* strains (Fig. S1). One *Lactobacillus* strain in particular, G5-1, was shown to absorb FFAs strongly (Fig. S1). Microscopic observations and molecular identification by 16S rDNA sequence analysis identified this strain as an unknown subspecies of *Lactobacillus reuteri* (Fig. 3); thus, it was named *Lactobacillus* JBD301 and deposited into the Korean Collection for Type Cultures (KCTC 12606BP).

FFAs-absorbing *Lactobacillus* JBD301 lowered the intestinal FFAs concentrations and thus reduced absorption of FFAs by host whereas increased fecal excretion of FFAs

It was shown that FFAs-absorbing *Lactobacillus* JBD301 was able to absorb FFAs up to 10 times more than other *Lactobacillus* (Fig. 4A, Fig. S1). Next, we investigated whether *Lactobacillus* JBD301 can reduce the absorption of FFAs into host as in the case of the mutant strains. The FFAs absorptions by *Lactobacillus* JBD301 and resulting removals of FFAs from absorbable pool of intestinal FFAs was determined by measuring the FFAs concentration in the gut fluid content where absorption of most FFAs occurs (Fig. 4B). In C57BL/6 mice colonized with *Lactobacillus* JBD301, the FFAs concentrations in fluid contents at small intestine were reduced to 69% of control whereas the

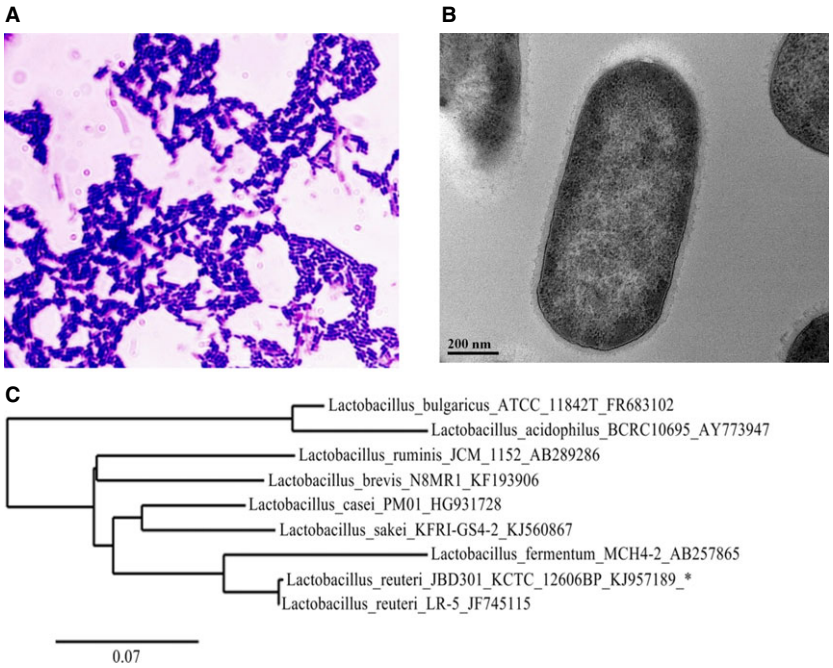

Fig. 3. The morphological and molecular characterization of natural FFAs-absorbing *Lactobacillus* strain, *L. reuteri* JBD301. (A) Gram staining of *Lactobacillus* JBD301. (B) TEM images of *Lactobacillus* JBD301. (C) Phylogenic tree of *Lactobacillus* JBD301. Accession numbers of bacterial strains are marked by an underscore next to the strain number. The phylogenetic tree was constructed by Phylogeny.fr set to build maximum-likelihood phylogenetic trees (PhyML). The scale bar represents an evolutionary distance.

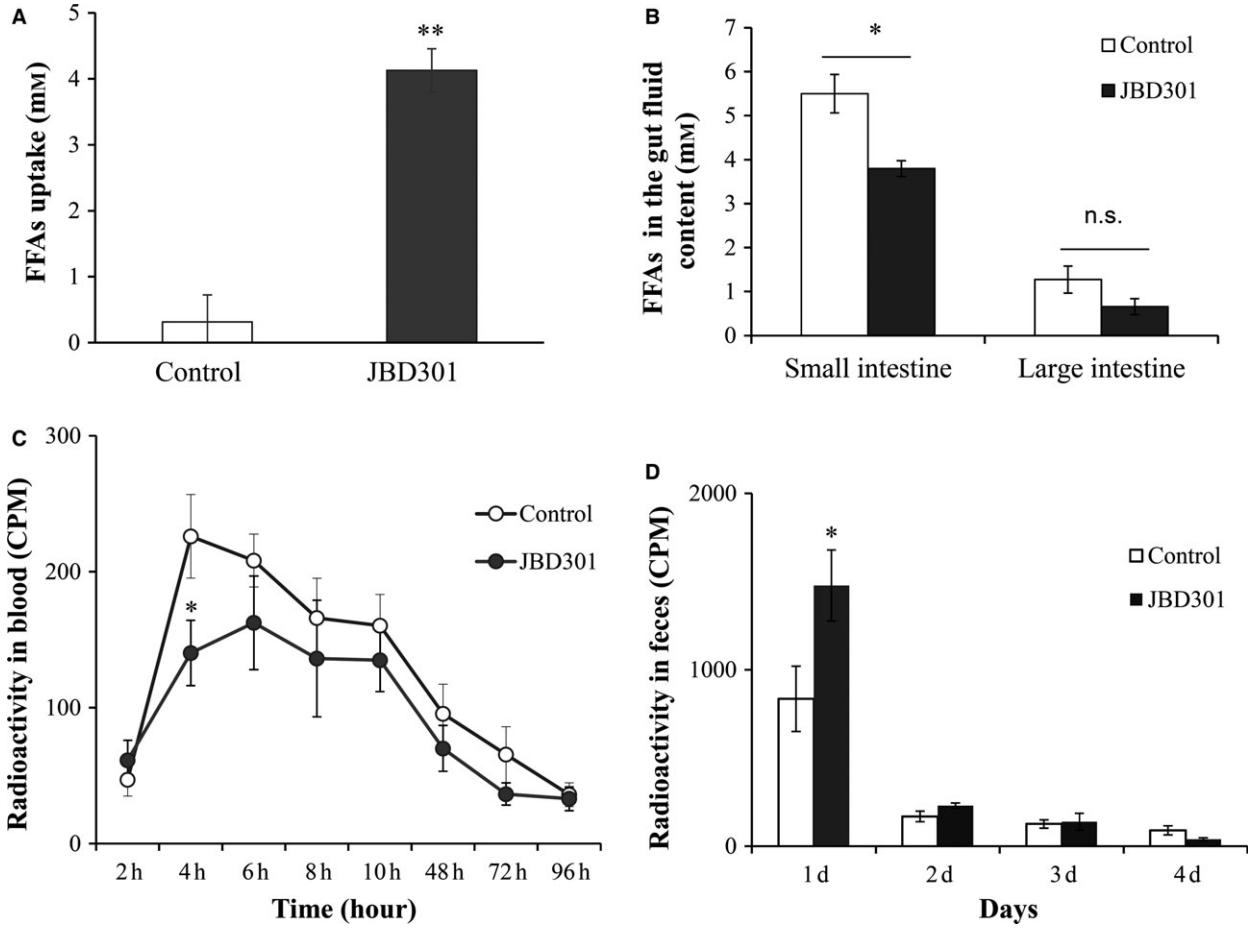

Fig. 4. The FFAs-absorbing *Lactobacillus* JBD301 lowered the intestinal FFAs concentration and limited the absorbable FFAs quantities into host, inhibiting absorption of FFAs by host but promoting excretion of FFAs. (A) The FFAs absorption by *Lactobacillus* JBD301 was compared with common *Lactobacillus* strain by measuring the FFAs concentration in the conditioned media. Values are mean ± SD ($n = 5$). (B) The intestinal FFAs concentrations in the gut fluid of JBD301-fed host was determined. Seven-week-old female C57BL/6 mice were randomized and fed a HFD only (control) or a HFD supplemented daily with 10^7 CFU of JBD301. After 3 weeks of administration, gut fluid contents were analyzed for total FFAs concentration. Values are mean ± SEM ($n = 3$). (C) The FFAs absorption from dietary fat was determined by measuring the radioactivity in the blood of JBD301-fed host. Seven-week-old female C57BL/6 mice were fed a HFD only (control) or a HFD supplemented daily with 10^7 CFU of JBD301. After 3 weeks of administration, feed containing radiolabeled fat, ^{14}C-triolein, was administered and radioactivities of the blood samples were analyzed. Values are mean ± SEM ($n = 7$). (D) The FFAs excretion was determined by measuring the radioactivity in the feces of JBD301-fed host. Seven-week-old female C57BL/6 mice were randomized and fed a HFD only (control) or a HFD diet supplemented daily with 10^7 CFU of JBD301. After 3 weeks of administration, feed containing radiolabeled fat, ^{14}C-triolein, was administered and radioactivities of the feces were analyzed. Values are mean ± SEM ($n = 7$). Statistical significance is shown as $*P < 0.05$ and $**P < 0.01$ versus control by ANOVA.

FFAs concentrations at large intestine were not much different. The changes in absorption and excretion of dietary fat in the *Lactobacillus* JBD301-fed host were also determined (Fig. 4C and D). After 3-week administration of *Lactobacillus* JBD301, radiolabeled dietary fat, ^{14}C-triolein, was orally administered to the mice. The radioactivities from absorbed fat in the blood of *Lactobacillus* JBD301-fed host were significantly decreased, down to 62% of control at 4 h after intake of radiolabeled food (Fig. 4C). The changes in the amount of excreted dietary fats, which were absorbed into *Lactobacillus* JBD301, were also measured by quantitating the radioactivities in the feces of *Lactobacillus* JBD301-fed mice (Fig. 4D). Compared to the unfed control, the radioactivities in the feces of JBD301-fed host were significantly increased, up to 176% of the control after 1 day from radiolabeled food intake.

FFAs-absorbing *Lactobacillus* JBD301 inhibited weight gain in host, both in mice and humans

In accordance with observed inhibition of FFAs absorption, administration of FFAs-absorbing *Lactobacillus* JBD301 resulted in a significant decrease in body weight of host animal (Fig. 5A). After 4 weeks, the body weight of control groups was 32.7 g with 4.3 g of weight gain. In contrast, JBD301 group was 28.1 g with 0.2 g of weight gain and orlistat group was 27.3 g with 0.8 g of weight loss. The most significant finding in this experiment was that the degree of body weight reduction by *Lactobacillus* JBD301 was as much as that of the mice that had been administered a pharmaceutically effective dose of orlistat.

After confirming that the degree of weight loss by *Lactobacillus* JBD301 could be up to that of a current pharmaceutical drug, a phase 2 clinical trial was conducted to determine the efficacy of *Lactobacillus* JBD301 in obese adults with $25 \leq \text{BMI} < 35$ (Fig. S2). This trial was registered with Clinical Research Information Service of Korea as KCT0000452 (https://cris.nih.go.kr). Recruited subjects were included in the trial after screening with inclusion and exclusion criteria. Randomized subjects were daily administered a 450 mg capsule containing either *Lactobacillus* JBD301 at 10^9 CFU/capsule as experimental ingredient or non-dairy creamer as placebo for 12 weeks. In this study, subgroup was analyzed in which 37 subjects received double-blinded materials which contain either the non-dairy creamer as placebo ($n = 19$) or *Lactobacillus* JBD301 as the experimental treatment ($n = 18$) (Fig. S3 and Table S4).

Without any dietary restrictions or additional exercise, changes in the body weight from baseline to endpoint were 0.31% (0.21 kg) in the *Lactobacillus* JBD301 group and 1.77% (1.45 kg) in the placebo group, resulting in a 1.46% (1.24 kg) between-group difference (Table S5). A Mann–Whitney *U* test showed that there was a statistically significant difference in the percentage change in weight between the *Lactobacillus* JBD301 and the placebo group ($P = 0.026$) as well as in the BMI ($P = 0.036$) from the 0-week assessment to the 12-week assessment (Table S5). A Wilcoxon signed rank test also confirmed that there was a statistically significant difference in the pairwise comparison of the percentage of the control between 0 and 12 weeks ($P = 0.028$) (Fig. 5B and Table S6). There were no adverse events related to the treatment.

Because the body weight of humans is heavily affected by food intake and lifestyle, and thus, the body weight of an obese person fluctuates, between-group differences, *i.e.* weight differences between the control and the experimental group, could be a more important parameter than the value of body weight

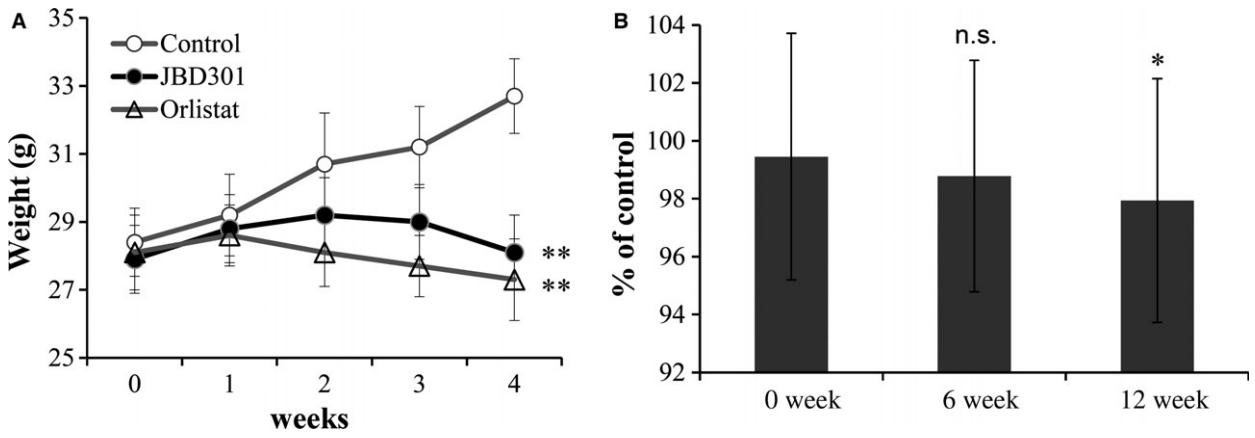

Fig. 5. Administration of the FFAs-absorbing *Lactobacillus* JBD301 significantly reduced body weight of the colonized hosts in both animals and humans. The diet-induced obese C57BL/6 mice were administered the HFD control diet (○) or the HFD supplemented with *Lactobacillus* JBD301 (●) or orlistat (Δ) daily. Seven-week-old female C57BL/6 mice were randomized and fed a HFD for 12 weeks to induce obesity. After 12 weeks on the HFD, administration, the animals were randomly divided into groups that received HFD only (control), HFD supplemented with *Lactobacillus* JBD301 (1×10^7 CFU·day^{-1}), or HFD supplemented with orlistat (Xenical® at 50 mg·kg^{-1} diet). (A) Body weights were monitored weekly. Values are mean ± SD ($n = 5$). Statistical significance is shown as $^{**}P < 0.01$ versus control by ANOVA. (B) A random, double-blind, placebo-controlled human study was conducted to determine the anti-obesity efficacy of *Lactobacillus* JBD301 in adults with $25 \leq \text{BMI} < 35$. Placebo group ($n = 19$) were administered single capsule of vegetable cream as control while experimental group ($n = 18$) were administered *Lactobacillus* JBD301 at dose of 1×10^9 CFU·day^{-1} for 12 weeks. Values are mean ± SD. Statistical significance of clinical data, body weight at 12 weeks, is shown as $^{*}P < 0.05$ versus control by Wilcoxon signed rank test ($P = 0.028$).

reduction in obesity clinical trials. In our clinical trial, the weight difference between the placebo and FFAs-absorbing *Lactobacillus* JBD301 groups was 1.24 kg without calorie restriction, which is far greater than that of the orlistat trial, where 0.71 kg was the weight difference compared to placebo after 180 mg of orlistat for 12 weeks under a hypocaloric diet [33]. Therefore, this work shows that the *Lactobacillus* strains with the ability to actively remove intestinal FFAs have anti-obesity effects as great as the most popular anti-obesity pharmaceutical drug, orlistat, in animal experiments as well as in a clinical trial.

However, abdominal fat, blood lipid profile, fasting blood glucose, blood insulin,= and HbA1c failed to show significant difference between placebo and experimental group in this trial. Further clinical trials need to consider more numbers of subjects with longer periods of intervention, if possible, with strict control of diet and exercise.

Discussion

Excess caloric intake from dietary fat is the most important determining factor for obesity, which has become more prevalent throughout the developed world [34,35]. For the vast majority of humans, caloric intake that exceeds caloric expenditure by as little as 1% could result in the accumulation of body fat and eventual obesity [36]. Therefore, the 30% reduction in FFAs absorption by orlistat produces significant weight loss [37].

In this work, we demonstrated that the *Lactobacillus* strains that lower intestinal FFAs concentration in the guts show anti-obesity effects almost as much as the most popular anti-obesity pharmaceutical drug, orlistat, in animal experiments as well as in a clinical trial (Fig. 5). Although orlistat inhibits FFAs generation for absorption, JBD301 absorbs FFAs and lowers FFA concentration in the gut fluid contents and thus reduces the amount of FFAs available for absorption. Eventually, they both reduce the FFAs uptake into the body and, thus can result in significant weight loss. In addition, *Lactobacillus* JBD301 strains have obvious advantages over the current pharmaceuticals for obesity. The *Lactobacillus* strains do not act on the brain but instead act peripherally and, therefore, have a superior risk-benefit profile to the current centrally acting drugs. Second, *Lactobacillus* does not act on lipid hydrolysis as orlistat does, which can cause unavoidable side effects in the GI tract, such as anal leakage and oily spotting. Third, *Lactobacillus* is a beneficial probiotic that conveys considerable safety as a drug for long-term administration.

Despite numerous studies, the direct impact of intestinal microbiota at the genus and species levels on the body weight of host remains unclear until now. Some *Lactobacillus* species are associated with weight gain whereas others are associated with weight loss [38,39]. For instance, administration of *L. acidophillus, L. fermentum* and *L. ingluviei* was associated with weight gain, whereas *L. plantarum* and *L. gasseri* were associated with weight loss. More complicatedly, different strains from the same species often showed different effects on weight [38]. Although some strains of *L. reuteri* were associated with obesity, other strains of *L. reuteri* prevent diet-induced obesity in a strain-dependent fashion [39,40]. In this study, we showed that lactobacilli either acquired or naturally having the ability to remove FFAs in the GI tract showed anti-obesity phenotypes, which implies that metabolic activities rather than microbial composition in the intestinal microbiota play determining roles in host phenotypes. These results were further supported by the recent finding that administration of *L. gasseri* SBT2055, a *Lactobacillus* strain having anti-obesity phenotype, decreased significantly the serum concentration of FFAs in hyperglycemic subject [41]. Considering that microbiota seems to affect host obesity by modulating nutrient uptake and energy metabolism, lactobacilli regulating intestinal absorption of FFAs could be the ideal approaches for anti-obesity phenotypes [42–44].

Recently, it has been acknowledged that the intestinal microbiota is associated with various human diseases, including obesity, diabetes, metabolic syndrome, inflammatory bowel disease, cognitive functions, cholelithiasis and autism [45–47]. Metagenomic studies demonstrated that the composition of the intestinal microbiota differs in control and disease groups in humans as well as animals [48–51]. However, investigations into the microbial species responsible for diseases have frequently produced conflicting results with no successful attempts to develop a treatment for these diseases by modulating the composition of the intestinal microbiota [52,53]. One possible explanation for the inconsistent correlation between the composition of the intestinal microbiota and the resultant diseases might be due to the intraspecies heterogeneity of bacterial genomes [54]. Even within a species, bacterial genomes are highly heterogeneous because of their haploid genomes and the presence of extrachromosomal elements; thus, metabolic activities within a species of bacteria frequently differ from each other [55]. To elucidate the determining role of the intestinal microbiota in obesity, based on our results, it is reasonable to investigate the metabolic differences of the intestinal microbiota rather than the compositional differences.

We also believe that this approach will lead to the development of living microbial drugs to treat various diseases associated with microbiota.

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Author contributions

S.T.H. and H.J.K. designed research; H.J.C., J.G.Y., I.A.L., M.J.L., Y.F.S., S.P.S., M.A.H.M.J. and J.H.Y. performed research; S.T.H., H.J.K., H.J.C. and J.G.Y. analyzed data; and S.T.H. and H.J.K. wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:
Fig. S1. Identification of natural *Lactobacillus* strains that strongly absorb FFAs.
Fig. S2. Study protocol for randomized controlled trial of effects of *Lactobacillus* JBD301 on obesity.
Fig. S3. Flow chart for randomized controlled trial of effects of *Lactobacillus* JBD301on weight loss in obesity.
Table S1. The acidification characteristics of milk by *L. acidophilus* KCTC 3179, mut1 and mut2.
Table S2. Confirmation of colonized *L. acidophilus* KCTC 3179, mut1 and mut2 in the GI tract of rats.
Table S3. Confirmation of colonized *L. acidophilus* KCTC 3179, mut1 and mut2 in the GI tract of the diet-induced obese rats.
Table S4. Study participants' baseline characteristics.
Table S5. Comparison of % change in outcome variables at 12th week between the placebo control and the experimental *Lactobacillus* JBD301 groups.
Table S6. Comparison of % control in body weight between 0 and 12th week.
Data S1. Methods.

nutrients

MDPI

Article

Body Fat Reduction Effect of *Bifidobacterium breve* B-3: A Randomized, Double-Blind, Placebo Comparative Clinical Trial

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Abstract: This double-blind, randomized clinical trial aimed to evaluate the efficacy and safety of *Bifidobacterium breve* B-3 (BB-3) for reducing body fat. Healthy individuals were randomized into the BB-3 or placebo group (1:1). Dual-energy X-ray absorptiometry was used to evaluate body fat reduction objectively. In the BB-3 group, body weight was lower than before BB-3 ingestion. Regarding waist circumference, hip circumference, and waist/hip circumference ratio, waist circumference and hip circumference were lower in the BB-3 group than in the placebo group at 12 weeks; the waist/hip circumference ratio was found to decrease at each visit in the BB-3 group, although there was no significant difference in the amount of change after 12 weeks. BB-3 did not cause any severe adverse reactions. Body fat was significantly lower in the BB-3 group than in the placebo group. In conclusion, ingesting BB-3 significantly reduces body weight, waist circumference, and hip circumference. Thus, BB-3 is safe and effective for reducing body fat.

Keywords: *Bifidobacterium breve* B-3; placebo-controlled study; randomized trial; obesity; nutritional supplement

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1. Introduction

The recent obesity development theory is explained by the "carbohydrate-insulin model" (CIM) based on the hormonal response to highly processed carbohydrates rather than the "energy balance model" (EBM) theory, which posits that obesity occurs because energy intake is less than consumption [1,2]. In 2015, 107.7 and 603.7 million children and adults, respectively, were identified to be obese. The prevalence of obesity since 1980 has doubled in more than 70 countries and has continuously increased in most other countries [3]. From 1990 to 2017, the global deaths and disability-adjusted life years (DALYs) attributable to a high body mass index (BMI) have more than doubled for both females and males [4]. The Global Burden of Disease Study level 3 causes of DALYs associated with high BMI in 2017 were ischemic heart disease, stroke, diabetes mellitus, chronic kidney disease, hypertensive heart disease, and low back pain [4]. Obesity is also associated with inherent complications and various chronic diseases such as type 2 diabetes, high blood pressure, hyperlipidemia, arteriosclerosis, stroke, osteoarthritis, and obstructive sleep apnea. Obesity was reported to directly cause 80% of diabetes cases and 20% of heart disease cases worldwide [5,6]. Given the increasing prevalence of various cancers, a cure for obesity has gathered great interest [7,8]. The World Health Organization has recognized obesity as a global health problem that needs to be treated due to its increasing prevalence. Obesity is a complex disease mainly caused by excessive caloric intake and lack of exercise, although social, genetic, and environmental factors also affect its occurrence [9,10].

Recent studies have suggested that gut microbiota is a factor that may influence obesity. It has also been found to support the ability to regulate energy balance, fat storage, neurohormonal function, and the immune system [11–14]. Moreover, altering the composition of the gut microbial ecosystem has been proposed as a novel approach to treating obesity. This strategy mainly involves altering the composition of the gut microbiome in obese individuals by ingestion of beneficial microorganisms, i.e., probiotics [15]. When consumed as a functional food ingredient, probiotics have been recognized to help lactic acid bacterial growth, suppress harmful bacteria, and facilitate good bowel movement. They have also been recognized for their benefits in vaginal health, immunity regulation, intestinal health, skin protection against further ultraviolet damage, and skin moisture. Several previous studies have reported the effect of bifidobacteria on the improvement of weight or body fat-related indicators [16]. Among them, *Bifidobacterium breve* B-3 (BB-3) is a strain with a patent for its body-fat reduction action. A non-clinical test conducted on mice showed the efficacy of BB-3 against obesity [17].

This study aimed to confirm the effectiveness of BB-3 in reducing body fat in Koreans.

2. Materials and Methods

2.1. Study Design and Participants

This randomized, double-blind, placebo comparative clinical trial (ver. 0.2, issue date 2021) recruited healthy individuals through a written notice posted on the hospital homepage and bulletin board of Semyung University Korean Medicine Hospital (Jecheon, Chungcheongbuk-do, Republic of Korea) until the target sample size was reached. Individuals willing to participate visited the Department of Internal Medicine and were screened according to the participant selection criteria presented in Table 1. The first participant was enrolled in April 2021. The total time for study participation was approximately 14 weeks, including a maximum of a 2-week wash-out period and safety assessment 2 weeks after the last visit. If a participant had a drug history of concomitant use of prohibited medication or food, a maximum 21-day wash-out period was required.

Table 1. Participant selection criteria.

Inclusion Criteria
(1) Age 19–60 years
(2) Body mass index (BMI) of $\geq$25 kg/m^2 and <30 kg/m^2
(3) Able to provide written informed consent
Exclusion Criteria
(1) Severe cerebrovascular disease (cerebral infarction and cerebral hemorrhage), heart disease (angina pectoris, myocardial infarction, heart failure, and arrhythmia requiring treatment), or malignant tumor within the last six months. However, participants with a medical history of cerebrovascular disease or heart disease who were clinically stable could participate in the trial at the investigator's discretion
(2) Taking drugs that affected body weight (fat absorption inhibitors and appetite suppressants, health food/supplements related to obesity, psychiatric drugs such as depression, beta-blockers, diuretics, contraceptives, steroids, and female hormones) within the last month
(3) Obese or overweight due to endocrine diseases such as hypothyroidism and Cushing's syndrome
(4) Maintenance treatment for gastrointestinal disorders (gastric ulcer, chronic digestive disorder, and irritable bowel syndrome)
(5) Psychologically significant medical history or current disease (schizophrenia, epilepsy, anorexia, and bulimia) or a history of alcohol and other drug abuse
(6) Judgment of inability to exercise due to musculoskeletal disorders
(7) Fasting blood sugar of $\geq$126 mg/dL, random blood sugar of $\geq$200 mg/dL, or patients with diabetes taking oral hypoglycemic agents or insulin

Table 1. *Cont.*

(8) Uncontrolled hypertension (blood pressure >160/100 mmHg measured after a 10-min rest)
(9) Alanine aminotransferase(AST) or Alkaline phosphatase(ALT) level at least 2.5 times higher than the laboratory's upper limit of normal
(10) Creatinine levels more than twice the upper limit of normal in the testing institute
(11) Weight loss ≥5% within the last three months
(12) Participation in a commercial obesity program within the last three months
(13) Participation in an obesity clinical trial within the last six months
(14) Pregnancy, lactation, or was planning to become pregnant during the study period
(15) An allergic reaction to the food study drug
(16) Others were considered unsuitable for the study at the discretion of the principal investigator
(17) The intake of probiotics within the last month

Participants in the study were randomly classified into treatment and placebo groups on the second visit (within 3 weeks of the first visit), which served as the baseline time point.

The inclusion and exclusion criteria were rechecked before randomization, and participants who met the criteria were enrolled. A baseline assessment was performed, and 33-day supplies of the investigational product or placebo were provided to the participants. Follow-up visits occurred 28 (visit 3), 56 (visit 4), and 84 (visit 5) days after the baseline assessment (visit 2). In addition, a 5-day visit window was allowed. Vital signs, medical history/concomitant drug examinations, and efficacy and safety evaluations were performed during visits 3–5 (Figure 1). Laboratory and pregnancy tests were performed at visits 1 and 5. Participants were notified of the hospital visit schedules by the clinical trial investigator.

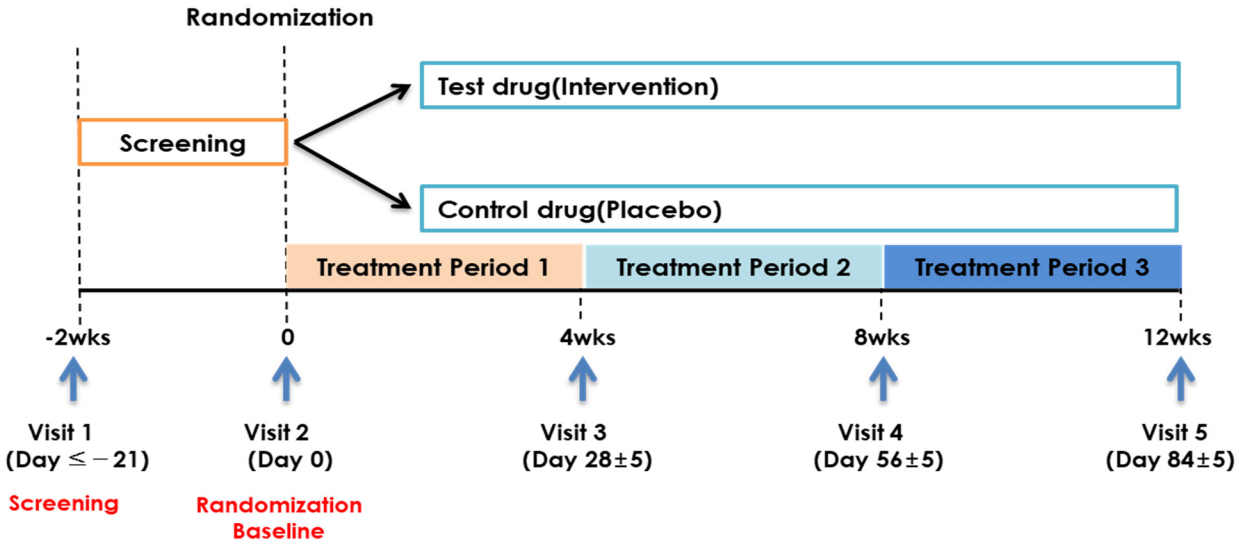

Figure 1. Clinical trial timeline.

2.2. Intervention

The investigational product contained BB-3 as the main ingredient and 50 mg (11.11%) maltodextrin (85.89%), magnesium stearate (1.0%), and silicon dioxide (2.0%) at 450 mg/cap as excipients. The placebo drug contained maltodextrin (97.0%), magnesium stearate (1.0%), and silicon dioxide (2.0%) as excipients at 450 mg/cap. BB-3 contained 1×10^{11} colony-forming units (CFUs) of BB-3 per 1 g of corn starch, including BB-3 as the same raw material as the B-3-EX product sold commercially by Morinaga Dairy in Japan. The daily intake of BB-3 in the BB-3 group was 5 billion CFU/capsule/day (Table S1). The test and placebo capsules were manufactured to be similar in shape, size, and color (Figure 2). The test

capsules disintegrated in the stomach. Test or placebo drugs were ingested orally once daily for 12 weeks. Participants were prescribed 1 month's dose at visit 2, visit 3, and visit 4 and were then encouraged to continue with the prescribed dose. The remaining unused capsules were returned at visit 2, visit 3, and visit 4 and counted to evaluate drug compliance.

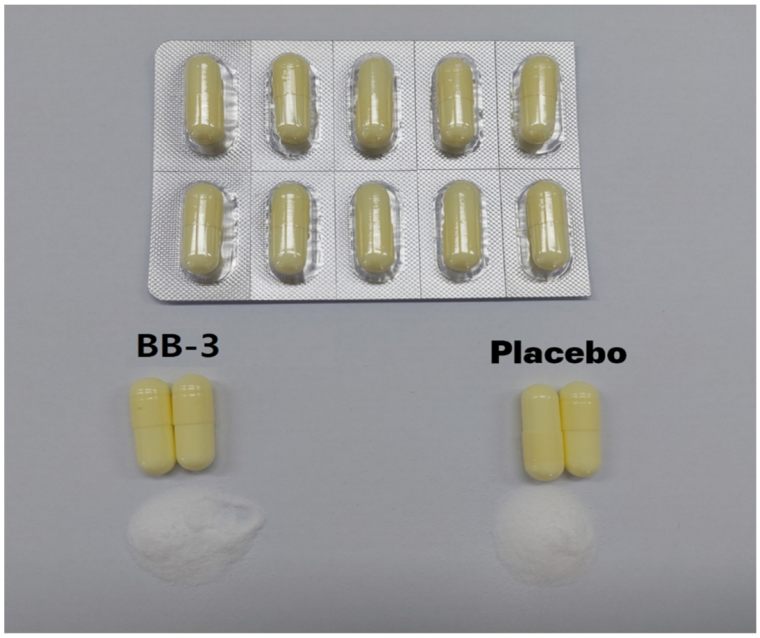

Figure 2. BB-3 and placebo capsules. BB-3: *Bifidobacterium breve* B-3.

The participants were guided to maintain their usual diet and exercise during the study period. However, they were banned from taking drugs or foods that could cause body fat loss. Drugs and food consumption, exercise activities, and diets followed before participation were allowed at the researcher's discretion. Information about all medications, including the items or names, doses, and duration of the medications taken, was recorded at each visit. The intervention was interrupted under the following conditions: a serious adverse event, use of a drug or undergoing a physical procedure that could affect body fat and lipid levels, participants wanting to stop participating in the study, difficulties in the evaluation due to administrative reasons (e.g., violation of dosing method or visit schedule), and difficulties in follow-up due to participants' personal reasons.

2.3. Randomization and Blinding

Stratified block randomization was performed. The participants were randomized to the placebo or experimental group in a 1:1 ratio. Using the SAS® system's randomization program, a random number sequence was created, starting with participant number 1. When packing food, the sponsor attached the food label for clinical trials according to the IP code list and supplied it to the test institution before the commencement of this clinical trial. The stratified block randomization method was used to prevent bias that could be involved in the allocation of intake groups, to increase comparability between groups, and to ensure balanced allocation. Stratified block randomization was performed at visit 2 according to sex (male and female). Randomization was performed using the web-based interactive web response system (IWRS), and the randomization code was reproducible by assigning a seed.

The randomization code and IP number were managed by a third-party individual unblinded to the data. The code and number were not disclosed until statistical analysis, except in cases where it was necessary to read the code owing to a serious medical emergency. The IP manager (or pharmacist) supplied the intervention for the clinical trial with

an IP number assigned to the participant. In case of defect or damage to the intervention, another IP number was reassigned using the IWRS system to maintain the treatment arm. To maintain double blinding (all researchers and subjects participating in clinical trials), participant allocation details and serious adverse reactions and codes mentioned in the production, packaging, and labeling of products used in the clinical trial were sealed in an envelope by the person in charge of the trial. The code was not released until the end of the study, except in inevitable cases where the code needed to be checked. Clinical trial sponsors provided interventions that matched the registration number assigned to the selected participants.

2.4. Endpoints

The primary endpoint was the change in body fat mass (g) and body fat percentage (%) assessed on dual-energy X-ray absorptiometry (DEXA) at 12 weeks from baseline. The secondary endpoints were as follows: (1) changes in lean mass (g), body fat mass (g) by area (arms, legs, trunk, android, and gynoid), body fat percentage (%) by area (arms, legs, trunk, android, and gynoid), and lean mass (g) by area (arms, legs, trunk, android, and gynoid) assessed using DEXA at 12 weeks from baseline; (2) changes in total fat area, subcutaneous fat area, visceral fat area, and visceral fat area/subcutaneous fat area ratio measured using abdominal computed tomography (CT) 12 weeks from baseline; (3) changes in body weight, body mass index (BMI), waist circumference, hip circumference, waist/hip circumference ratio at 4, 8, and 12 weeks from baseline; (4) changes in blood lipid concentrations (total cholesterol, low-density lipoprotein (LDL)-cholesterol, triglyceride (TG), and high-density lipoprotein (HDL)-cholesterol), leptin, and adiponectin at 12 weeks from baseline. As the primary outcome measure, DEXA was used to assess the body fat mass and percentage at baseline and week 12.

Total fat mass was measured in a supine position using a LUNAR Prodigy Vision scanner (software version 6.70; General Electric Medical Systems, Madison, WI, USA) and a whole-body DEXA scanner. Total fat mass and body fat mass were obtained using standard soft tissue measurement methods. Body fat mass was calculated as the amount of fat present in the section (chest, abdomen, and pelvis) surrounded by the virtual boundary line that separates the head and limbs when measured with a whole-body DEXA scanner. Abdominal CT was used to measure visceral fat area, subcutaneous fat area, total abdominal fat area, and visceral fat/subcutaneous fat area ratio at baseline and week 12. A CT scan was performed between the fourth and fifth lumbar vertebrae. The high accuracy of CT makes it the preferred method for measuring visceral and subcutaneous fat [18]. Body weight and BMI were measured at baseline and weeks 4, 8, and 12. Waist circumference, hip circumference, and waist circumference/hip circumference ratio were assessed at baseline and weeks 4, 8, and 12. Following the World Health Organization guidelines, waist circumference was measured at the midpoint between the lower margin of the last palpable rib in the midaxillary line and the top of the iliac crest. Hip circumference was measured at the largest circumference of the buttocks (World Health Organization. Waist circumference and waist-hip ratio: Report of a WHO expert consultation. Geneva, Switzerland: WHO Press; 2011).

Serum lipid (total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol) and adipocytokine (leptin and adiponectin) concentrations were assessed at visit 1 and visit 5. Blood samples were collected after at least an 8-h fast and analyzed in the laboratory. Physical activity and dietary habits (24-h recall) were surveyed. Physical activity was evaluated at visits 2 and 5 using the Korean version of the short-form International Physical Activity Questionnaire (IPAQ). The IPAQ was selected as a questionnaire tool for various surveys conducted by the WHO; reliability validity studies were conducted in 12 countries and it is currently being used worldwide [19]. Through the IPAQ, participants were asked to recall and record the amount of activity for the week before visits 2 and 5. Dietary habits were analyzed using the 24-h recall method. A 24-h recall diary was prepared at visits 2 and 5. In this study, the participant's diet was

investigated through total calorie (kcal) analysis using the CAN-PRO program (CAN 5.0 Web ver.).

2.5. Safety

Adverse reactions and side effects were evaluated through interviews during the visit or through blood and urine tests before and after the intervention.

2.6. Sample Size Calculation

We referred to the study by Cho et al. [20], who reported that the change in body fat mass of the herbal extract powder (Imperata cylindrical Beauvois, Citrus unshiu Markovich, and Evodia officinalis Dode) group was −1.6 kg, the change in body fat mass of the placebo group was −0.1 kg, and the unpaired *t*-test *p*-value between the two groups was 0.023. Based on these results, it was assumed that the effect size of this clinical trial was −1.5, and the pooled standard deviation was 2.2676 kg. Based on the change in body fat mass, which was calculated for the number of participants, the number of participants required to achieve a significance level of 5% and power of 84% was calculated to be 40 participants per group. Considering a dropout rate of 20%, 50 participants per group (=40/(1 − 0.2)) for a total of 100 participants were planned to be enrolled.

2.7. Statistical Analyses

A statistical hypothesis test was conducted at a two-sided significance level of 0.05. The study endpoints were analyzed using the number of participants, mean, and standard deviation. Furthermore, normally distributed data were analyzed using covariate analysis with baseline values and sex as the covariate. Non-normally distributed data were compared between groups using the Wilcoxon rank sum test. Continuous variables were reported as the mean and standard deviation and analyzed using the two-sample *t*-test or Wilcoxon rank sum test for inter-group comparison. Moreover, categorical variables were reported as frequencies and percentages and analyzed using Pearson's chi-square test or Fisher's exact test for inter-group comparison. Intra-group analysis was performed using paired *t*-tests or Wilcoxon signed-rank test. The per-protocol set was used to analyze the primary and secondary endpoints. The per-protocol set included all participants who completed the study protocol and had no major protocol deviations. The safety set included all participants who received at least one capsule of the investigational product and had at least one safety assessment; this set was used for the safety analysis. All statistical analyses were performed using SAS® software (version 9.4, SAS, Cary, NC, USA).

3. Results

3.1. Participant Characteristics

The first participant was screened on 6 April 2021 and the last was screened on 30 July 2021. In total, 104 participants were evaluated; among them, 4 participants were excluded, and finally, 100 participants (51 participants in the BB-3 group and 49 participants in the placebo group) were enrolled. Consequently, 6 participants from each group (total: 12) dropped out, and thus, 83 participants (42 participants in the BB-3 group and 41 participants in the placebo group) completed the clinical trial (Figure 3). There was no significant between-group difference in age (46.55 ± 9.76 years in the BB-3 group vs. 45.02 ± 9.23 years in the placebo group, $p = 0.3361$). There were also no significant between-group differences in sex, height, weight, BMI, waist/hip circumference, fat mass index, fat-free mass index, and family history of obesity (Table 2).

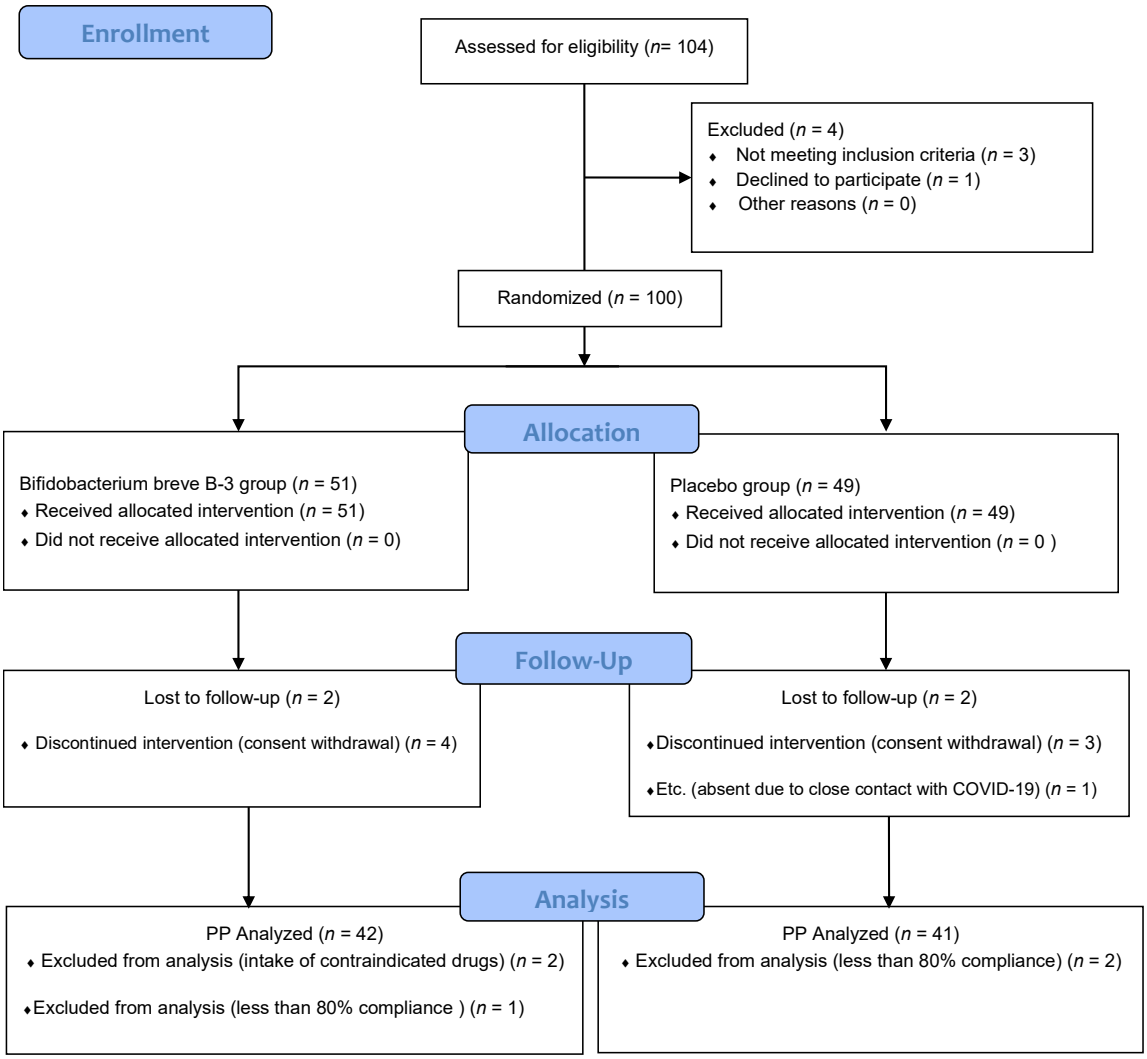

Figure 3. Trial flowchart. COVID-19: coronavirus disease 2019, PP: per protocol.

Table 2. Participant characteristics by group.

		BB-3 Group (n = 42)	Placebo Group (n = 41)	p-Value
Sex	Male	15 (35.71)	11 (26.83)	0.3829 [1]
	Female	27 (64.29)	30 (73.17)	
Age, years		46.55 ± 9.76	45.02 ± 9.23	0.3361 [2]
Height, cm		164.17 ± 9.15	162.22 ± 8.87	0.3343 [2]
Weight, kg		72.71 ± 8.22	70.82 ± 8.17	0.2948 [3]
Body mass index		26.93 ± 1.29	26.85 ± 1.38	0.7325 [2]
Waist circumference, cm		88.39 ± 4.58	87.62 ± 5.55	0.4928 [3]
Hip circumference, cm		99.04 ± 3.66	98.60 ± 3.89	0.6003 [3]
Fat mass index, g		25,446.90 ± 4405.20	26,163.00 ± 3809.42	0.4311 [3]
Fat-free mass index, g		47,094.50 ± 8707.45	44,334.07 ± 8532.53	0.0965 [2]
Family history of obesity	Yes	16 (38.10)	16 (39.02)	0.9307 [1]
	No	26 (61.90)	25 (60.98)	

[1] p-value for the chi-square test, [2] p-value for the Wilcoxon rank sum test, [3] p-value for the two-sample t-tests. Data are presented as n (%) or as the mean ± SD. Abbreviations: BB-3: *Bifidobacterium breve* B-3, SD: standard deviation.

3.2. Study Endpoints

The amount of body fat (g) was significantly lower after BB-3 intake than before (p = 0.0005). Meanwhile, in the placebo group, although body fat was lower after in-

take than before intake, the difference was not significant. Importantly, the amount of body fat was significantly lower in the BB-3 group than in the placebo group ($p = 0.0170$) (Table 3). The amount of body fat percentage (%) was decreased in both groups; however, the differences within and between groups were not significant ($p = 0.3760$) (Table 3). Regarding secondary outcomes, weight, BMI, waist circumference, and hip circumference measured at visits 2 and 5 were significantly lower than those measured at baseline values (Tables S2 and S3). In the BB-3 and placebo groups, there were no significant differences in the other secondary endpoints; however, total cholesterol, low-density lipoprotein (LDL)-cholesterol, triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, and leptin, excluding adiponectin, showed a tendency to decrease after 12 weeks. Even so, there was no statistical significance between the two groups. (Tables S4–S6).

Table 3. DEXA results at 12 weeks from baseline according to the groups (PPS).

		BB-3 Group (*n* = 42)	**Placebo Group (*n* = 41)**
Body fat mass, g	V2	25,446.90 ± 4405.20	26,163.00 ± 3809.42
	V5	24,859.86 ± 4382.83	26,098.63 ± 4022.56
	V5-V2	−587.05 ± 1004.42	−64.37 ± 933.76
	p-value	0.0005 [1]	0.6613 [1]
	Difference V5-V2 (Tx-Px)	−522.68 ± 970.17	
	LS mean difference [5]	−528.56	
	p-value	0.0170 [3]	
Body fat percentage (%)	V2	36.60 ± 6.67	38.73 ± 6.14
	V5	36.28 ± 6.77	38.64 ± 6.25
	V5-V2	−0.32 ± 1.26	−0.09 ± 0.97
	p-value	0.1097 [1]	0.5431 [1]
	Difference V5-V2 (Tx-Px)	−0.22 ± 1.12	
	LS mean difference [5]	−0.23	
	p-value	0.3760 [3]	
Fat-free mass, g	V2	47,094.50 ± 8707.45	44,334.07 ± 8532.53
	V5	46,622.79 ± 8539.42	44,362.80 ± 8454.12
	V5-V2	−471.71 ± 1500.65	28.73 ± 840.49
	p-value	0.0916 [2]	0.8279 [1]
	Difference V5-V2 (Tx-Px)	−500.45 ± 1220.14	
	p-value	0.1172 [4]	

[1] *p*-value for the paired *t*-tests, [2] *p*-value for the Wilcoxon signed rank test, [3] *p*-value for ANCOVA adjusted for baseline values and sex, [4] *p*-value for the Wilcoxon rank sum test, [5] ANCOVA results adjusted for baseline values and sex. Tx: BB-3, Px: placebo, ANCOVA: analysis of covariance, PPS: per protocol set. Data are presented as the mean ± SD. Abbreviations: V2: Visit 2, V5: Visit 5.

3.3. Safety

In the BB-3 group, 15 (29.41%) participants had 21 adverse reactions; in the placebo group, 14 (28.57%) participants had 19 adverse reactions, with no significant between-group differences ($p = 0.9262$). The most common adverse reactions, such as muscle pain, headache, and injection site pain, occurred after COVID-19 vaccination. All other adverse reactions were mild and unrelated to the study drug and no serious adverse reactions occurred (Table S7). For the hematological test results, the red blood cell, hemoglobin, hematocrit, and platelet levels before and after ingestion of BB-3 were significantly higher than before ingestion of food for human application in both the groups; however, both the groups showed changes near the normal range. There was no significant between-group difference in the amount of change (Table S9). In the BB-3 group, the alanine aminotransferase (ALT) level was significantly higher after treatment than before, although the difference was not significant. There was also no significant between-group difference

with respect to the amount of change in ALT levels (Table S9). There were no significant changes in other laboratory parameters. There were also no significant normal/abnormal changes in the urine test results before/after treatment (Table S10).

3.4. Physical Activity and Diet

There were no significant between-group differences in the dietary intake before (0 weeks) and after (12 weeks) of the intervention. (Table S8).

4. Discussion

To evaluate the body fat reduction effect of BB-3, a randomized, double-blind clinical trial was conducted in overweight adults in which BB-3 or a placebo was administered for 12 weeks. At 12 weeks, body weight and BMI were significantly lower in the BB-3 group than in the placebo group. Out of the waist circumference, hip circumference, and waist/hip circumference ratio, waist circumference and hip circumference were lower in the BB-3 group than in the placebo group at 12 weeks.

The composition of the gut microbiota differs between lean and obese participants and is recognized as a therapeutic target of obesity. In a meta-analysis of randomized controlled trials to examine the effects of probiotic supplementation on body composition in overweight (BMI 25–30 kg/m^2) and obese (BMI $\geq$ 30 kg/m^2) participants, five studies reported changes in body fat percentage, and the pooled estimate showed that percent body fat was significantly lower in the intervention groups (-0.60%) than in the control groups, with low heterogeneity among the studies [21,22]. BB-3 is a promising anti-obesogenic strain.

Some studies have reported a dose-dependent inhibition of body weight gain and visceral fat deposition and improved serum levels of total cholesterol, glucose, and insulin with BB-3 administration in diet-induced obese mice [23]. In humans, the daily intake of capsules containing a lyophilized powder of BB-3 at a dose of 5×10^{10} CFUs/day reduced body fat mass [16]. In addition, several clinical trials have reported the positive effects of probiotic strains on reducing visceral fat. The 12-week consumption of fermented milk containing *Lactobacillus gasseri* SBT2055 significantly reduced visceral fat areas in adults with accumulated visceral fat (81.2–178.5 cm^2) [24]. Furthermore, the 12-week consumption of fermented milk containing *B. animalis* ssp. lactis GCL2505 was also recently reported to significantly reduce visceral fat areas in healthy participants with BMIs ranging between 23 kg/m^2 and 30 kg/m^2 [22,25].

In this clinical trial, the amount of body fat (g) assessed using DEXA, body weight, BMI, waist circumference, and hip circumference were significantly lower after BB-3 ingestion than before. Notably, similar to that in a previous study [22], visceral fat tended to be lower than baseline values in the BB-3 group, which seems to have affected the reduction in body fat in the android and trunk regions. In addition, although the reduction in body fat mass was effectively lower in the BB-3 group, the reduction in body fat percentage was not significant. However, the body fat percentage tended to decrease in the BB-3 group, particularly in the trunk and android areas. This is considered to be due to the decrease in visceral fat and indicates the body fat reduction effect of BB-3. Previous studies have shown that the probiotic strain *B. breve* B-3 increased the number of cells and the proportion of bifidobacteria in the intestine [22]. In addition, the upregulation of glucagon-like peptides and proglucagon expression, such as Fiaf in the intestine and adiponectin expression in the B-epiticular fat pad, has been shown to be effective in preventing obesity and insulin resistance [16].

Cani et al. [26] suggested that the regulation of intestinal peptides involved in the regulation of energy and glucose homeostasis could be one of the mechanisms involved in the improvement of the microbiota regulation of metabolic syndrome. They found that OFS administration increased colonic proglucagon, such as a glucagon-like peptide (GLP-2). GLP-1 and GLP-2 are produced and released by enteroendocrine L cells in the distal ileum and large intestine [27]. GLP-1 stimulates postprandial insulin secretion and reduces appetite by stimulating the hypothalamus and stomach. Studies of germ-free and

normal mice have shown that the microbiota promotes the absorption of monosaccharides from the intestinal lumen and, consequently, induces new liver adipogenesis [28]. The fasting-inducing adipocyte factor, Fiaf, is a member of the angiopoietin-like family of proteins that are repressed in the intestinal epithelium. Fiaf is also a circulating lipoprotein lipase inhibitor and its inhibition is essential for microbial-induced triglyceride deposition in adipocytes [29]. In previous studies, Fiaf expression was significantly upregulated in the small intestine of B-supplemented mice [16]. BB-3 affects the mechanisms involved with the reduction in fat accumulation in adipocytes.

Obesity and insulin resistance are factors associated with metabolic syndrome. Hypertrophic adipocytes produce abnormal adipokines and cytokines, such as TNF-alpha, MCP-1, FFA, IL-6, and resistin, which inhibit insulin signaling in hepatocytes and induce insulin resistance. Meanwhile, adiponectin in normal adipocytes has been shown to play an important role in regulating energy homeostasis and insulin sensitivity. Studies in adiponectin transgenic mice suggest that insulin resistance is associated with increased expression of molecules involved in fatty acid oxidation (e.g., acyl-CoA oxidase), and molecules involved in energy dissipation (e.g., dissociation of proteins 2 and 3) have been shown to be related to increased fatty acid oxidation in skeletal muscle [30]. Adiponectin expression was significantly upregulated in the epididymal fat pad of *B. breve* B-3-fed mice. These results suggest that the level of adiponectin upregulated by *B. breve* B-3 administration is involved in improving insulin resistance by preventing adipocyte hypertrophy [16].

This current clinical trial found that body fat and body weight were lower in the BB-3 group than in the control group, consistent with the results of previous clinical trials. Overall, this result supports the assertion that BB-3 administration induces a decrease in visceral fat that results in a decrease in body fat mass. Previous experimental studies showed that BB-3 intake affects fat cells or fat metabolism and has a body fat reduction effect. However, improvements in insulin resistance and related indicators still need to be investigated. This current clinical trial provides baseline evidence for future studies on the body fat reduction effect of BB-3 in humans. In this clinical trial, in the BB-3 group, there was a statistically significant decrease in body fat mass as well as a decrease in lean body mass, although not statistically significant. This decrease in body fat mass and lean mass produced a statistically significant weight loss effect. On the contrary, there was no significant decrease in body fat percentage. This is because, even though there was a statistically significant decrease in body fat mass, the decrease in body fat percentage was offset by the decrease in lean mass. For reference, the body fat percentage is composed of the ratio of body fat mass to muscle mass, and most of the lean mass is composed of muscle mass. Diet programs and functional food intake reduce both fat mass and lean body mass, resulting in weight loss. However, it is important to prevent a decrease in lean mass because it causes the weight to increase again (the yo-yo effect) [31]. Therefore, in order to maintain weight loss in the long term, it is important to incorporate appropriate exercises [32].

In this clinical trial, lifestyle factors, such as exercise and eating habits, were adjusted to remain the same during the test period and no special exercise prescription was administered. Intake of BB-3 resulted in a decrease in lean body mass along with a decrease in body fat mass that can occur with initial weight loss. This resulted in a statistically non-significant decrease in body fat percentage. In the future, if a long-term study that combines exercise prescription with the intake of BB-3 is conducted, it can be assumed that the effect of weight loss due to the reduction in body fat mass and percentage of body fat, without reduction in lean body mass, will be confirmed. The limitation of this study was that it was a single-center study and not a crossover study. Multicenter and crossover studies in the future will generate clearer and more reliable evidence. In addition, studies that can confirm changes in intestinal microbes after taking BB-3 and mechanistic studies on the effect of BB-3 on body fat reduction are considered necessary.

5. Conclusions

Body fat mass (g) was significantly lower after BB-3 intake. Meanwhile, although body fat percentage (%) and fat-free mass (g) also decreased, no significant changes were observed. The reduction in body fat mass was particularly affected by the reduction in body fat mass in the trunk and android regions. Body weight and BMI were lower after the intervention than before in the BB-3 group, with parameters showing a continuous decrease at every visit (weeks 4, 8, and 12). In the 12th week, body weight and BMI were significantly lower in the BB-3 group than in the placebo group. Although body fat mass was effectively reduced in the BB-3 group, there was no statistically significant change in body fat percentage in the placebo group. Waist- and hip-circumferences were significantly lower in the BB-3 group than in the placebo group at 12 weeks. The waist/hip circumference ratio also decreased at each visit in the BB-3 group but no significant change was observed at 12 weeks in the placebo group. Collectively, these findings indicate that BB-3 can safely and effectively reduce not only body fat but also body weight, waist circumference, and hip circumference. Future research should be conducted on the effects of BB-3 on the intestinal environment, its mechanisms, and the effects of hormone changes involved in human metabolism.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15010028/s1, Table S1: Raw materials and mixing ratio of the study intervention; Table S2: Changes in body weight and body mass index (PPS); Table S3: Changes in waist circumference, hip circumference, and waist-hip-ratio (PPS); Table S4: DEXA results by anatomical region at 12 weeks from baseline values (PPS); Table S5: CT results at 12 weeks from baseline values (PPS); Table S6: Results of blood lipid levels at 12 weeks from baseline values (PPS); Table S7: Adverse effects (SAS); Table S8: Changes in physical activity and dietary habits; Table S9; Blood test results (SAS); Table S10: Urine test results (SAS); Table S11: Abbreviations and glossary of terms.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of SEMYUNG UNIVERSITY KOREAN MEDICINE HOSPITAL (protocol code SMJOH-2021-03-01 and date of approval 24 March 2022). The study was registered at the Korean Clinical Research Information Service (KCT0006857).

Informed Consent Statement: Informed consent was obtained from all participants involved in the study.

Data Availability Statement: The data presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: Hyun Kyung Sung and Seon Mi Shin are employed by Semyung University; Sang Jun Youn and Yong Choi are employed by RnBS; Sang Won Eun is employed by Daehan Chemtech. All authors declare no other competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Review

Bifidobacterium animalis subsp. *lactis* 420 for Metabolic Health: Review of the Research

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Abstract: The growing worldwide epidemic of obesity and associated metabolic health comorbidities has resulted in an urgent need for safe and efficient nutritional solutions. The research linking obesity with gut microbiota dysbiosis has led to a hypothesis that certain bacterial strains could serve as probiotics helping in weight management and metabolic health. In the search for such strains, the effect of *Bifidobacterium animalis* subsp. *lactis* 420 (B420) on gut microbiota and metabolic health, and the mechanisms of actions, has been investigated in a variety of in vitro, pre-clinical, and clinical studies. In this review, we aim to highlight the research on B420 related to obesity, metabolic health, and the microbiota. Current research supports the hypothesis that gut dysbiosis leads to an imbalance in the inflammatory processes and loss of epithelial integrity. Bacterial components, like endotoxins, that leak out of the gut can invoke low-grade, chronic, and systemic inflammation. This imbalanced state is often referred to as metabolic endotoxemia. Scientific evidence indicates that B420 can slow down many of these detrimental processes via multiple signaling pathways, as supported by mechanistic in vitro and in vivo studies. We discuss the connection of these mechanisms to clinical evidence on the effect of B420 in controlling weight gain in overweight and obese subjects. The research further indicates that B420 may improve the epithelial integrity by rebalancing a dysbiotic state induced by an obesogenic diet, for example by increasing the prevalence of lean phenotype microbes such as *Akkermansia muciniphila*. We further discuss, in the context of delivering the health benefits of B420: the safety and technological aspects of the strain including genomic characterization, antibiotic resistance profiling, stability in the product, and survival of the live probiotic in the intestine. In summary, we conclude that the clinical and preclinical studies on metabolic health suggest that B420 may be a potential candidate in combating obesity; however, further clinical studies are needed.

Keywords: *Bifidobacterium lactis*; B420; gut microbiota; metabolic health; metabolic syndrome

1. Introduction

The past decades of research have enabled us to better understand the key role of different microbial populations in human health and disease. Our microbiota is not only remarkable in its abundance, but also in its impact on health, interacting continuously with our body and either sustaining health or causing disease, depending on the ecological function of the microbes [1].

The gastrointestinal tract harbors a vast number of bacteria (10^{13}), which roughly equals the number of cells that make up the human body [2]. Commensal gut bacteria are involved in many metabolic processes such as fermentation of undigested carbohydrates into short-chain fatty acids and other metabolites, digestion and absorption of nutrients, but also in the maturation of the immune

system, as well as providing protection against incoming, potentially pathogenic microbes. Research indicates that specific probiotic strains or their combinations could be used to restore or maintain the composition and activity to a "healthy" intestinal microbiota and, thus, reduce the risk for a range of diseases or unfavorable conditions [3,4]. Maintaining the ecological balance of the complex microbial community in the gastrointestinal tract has been associated with the development and maintenance of intestinal immune function and metabolic processes, as well as other physiological functions, making the microbiota a critical factor for general human wellbeing [5–7].

The origin of a microbial strain or its natural habitat (e.g., the human gastrointestinal tract) is not a guarantee or precondition of its performance as a probiotic from the efficacy, safety, technological, or application perspective. Instead, in addition to being "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [8], a probiotic strain should be proven safe by appropriate phenotypic and molecular techniques and/or toxicology studies for acute or chronic toxicity, be able to resist acid and bile to survive the upper gastrointestinal tract, have good technological properties to be produced at large scale, and survive in sufficient counts until the end of shelf life.

Bifidobacteria were discovered at the turn of 18th and 19th centuries by Tissier in the feces of breast-fed infants, and since then, *Bifidobacterium* spp. have been shown to be comprised of Gram-positive, non-spore forming, anaerobic, pleomorphic bacteria [9–11]. Bifidobacteria have been shown to represent one of the most abundant genera present in a healthy gut early in life, being the most abundant genus present in the intestine of healthy breastfed infants, and to play an important role in gut homeostasis and immune system development [12–14]. During late adulthood and in several diseases, the levels of *Bifidobacterium* spp. and its species diversity have been shown to decrease [15]. In general, and relative to the stage of life, a higher proportion of bifidobacteria in the intestinal tract is considered beneficial to health. Today, evidence has emerged to indicate the impact of many bifidobacteria on the host's immune system and metabolism, resulting in an association with a range of health benefits such as a reduced risk of respiratory tract infections and various gastrointestinal disorders and infections, particularly antibiotic associated diarrhea [15,16]. The only certain way to establish the true benefit of a probiotic strain is by systematic in vitro and in vivo studies and, in particular, randomized and placebo-controlled human intervention studies.

Bifidobacterium animalis subsp. *lactis* (*B. lactis*) is one of the most common *Bifidobacterium* species utilized as a probiotic in commercial products in North America and Europe. *B. lactis* has been used in fermented foods for decades and was scientifically classified by Meile et al. in 1997 [17], then re-classified as *B. animalis* subsp. *lactis* in 2004 [18]. However, for simplicity, we will here refer to the species as *Bifidobacterium lactis*.

One of the probiotic strains that has been studied for its mechanism of action and clinical benefits is *Bifidobacterium animalis* subsp. *lactis* 420 (B420). Recently, the complete genome sequence of B420 has been published, allowing for more stringent strain identity confirmation among other genetically similar *B. lactis* strains [19]. The health benefits that have been shown with B420 consumption include for example control of body fat mass gain in a human intervention trial [20]. Preclinical data furthermore suggest enhancement of mucosal integrity [21,22] and glycemic control [23], as well as improving host resistance to pathogens [24,25]. In this review, we will discuss the preclinical and clinical studies on B420 and the mechanisms of action of the associated health benefits, as well as the technological properties of B420 in the context of an industrial probiotic. The review is based on literature searches performed in PubMed, and the manuscript includes all published data on B420 and metabolic health published prior to February 2020.

2. B420 and Health Benefits

To date, probiotic interventions and mechanistic trials related to weight management and metabolic health have mainly focused on the genera *Lactobacillus* and *Bifidobacterium*. B420 has shown promise in weight maintenance in a randomized placebo-controlled clinical study, as well as inducing a better

metabolic health state in animal studies via glycemic control by reducing glucose levels and improving insulin sensitivity [20,23,26].

There is growing evidence on the ability of B420 to affect weight and metabolism via gut barrier function and gut microbiota composition modulation in various in vitro and in vivo trials, as well as in a clinical study. An important feature of B420 in weight maintenance can be related to its ability to enhance intestinal epithelial integrity in vitro [22] and in vivo [27]. Furthermore, in obesogenic mouse models, B420 feeding has been shown to decrease in the quantity of inflammatory markers and gut-derived bacteria in tissues [23,25].

The early findings obtained from in vitro and animal studies led to the hypothesis that B420 could reduce metabolic endotoxemia by improving gut barrier function, and hence, its consumption could lead to improvement in metabolic health, and consequently to reduced fat mass. This mechanistic hypothesis has been used in the design of the later in vivo and human intervention trials with B420. Based on current research, it seems that the benefits of B420 on metabolic health are associated with its ability to modulate the complex web of intertwined metabolic pathways.

2.1. Gut Microbiota Composition in Obesity

The differences in gut microbiota composition between obese and lean mice and humans was initially reported by Ley et al. (2005) and Turnbaugh et al. (2006) more than a decade ago [28,29]. Since then, early publications in cross-sectional studies about the differences between healthy and obese subjects have indicated that large phyla-level shifts, mainly as an increase in the *Firmicutes*/*Bacteroidetes* ratio, can correlate with obesity [30]. More recently, a higher *Firmicutes*/*Bacteroidetes* ratio of obese children compared to normal weight children has been reported, indicating early discordant shifts in microbial balance in childhood obesity [31,32].

However, there have been discrepancies between these findings, and as Walters et al. (2014) pinpointed in their meta-analysis, methodological differences make the comparison of various studies difficult [33]. Moreover, the methodological differences can start already at DNA extraction, which can yield 10 to 1000 fold differences depending on the method used and bacterial group studied [34]. Methodological variation is not, however, the only factor explaining the observed incoherence. Le Chatelier et al. (2013) showed that the microbiota compositional shifts are present only in certain subpopulations of obese individuals [35]. In these predisposed individuals, it was observed that 36 bacterial genera were less dominant, including *Faecalibacterium*, *Bifidobacterium*, *Lactobacillus*, and *Akkermansia*, among others, and the changes in microbiota composition were associated with metabolic disturbances [35,36]. It has also been shown in some studies that dietary intake correlates more strongly with changes in microbiota than body mass index does [37].

The variability in the diversity of microbial species causes differences between lean and obese individuals' energy balance by affecting the efficiency of energy harvest, as well as the storage capacity and utilization of the harvested energy [30]. Decreases in resting energy expenditure have been shown to coincide with an increase in the abundance of the *Firmicutes* phylum with a 20% increase corresponding to an increase of 150 kcal in energy harvest per day in humans [38]. Furthermore, it has been suggested that changes induced by probiotics on the microbiome affecting energy metabolism and appetite could shift an individual from a microbiota associated with an obese phenotype to that of a lean one, consequently altering the phenotype as well [39].

As a consequence of the above-mentioned ability of microbes to affect energy metabolism, the most important step in the mode of action of probiotics in metabolic health outcomes might well be inducing a beneficial shift in microbiota composition. The effect of B420 on microbiota composition was studied as part of a placebo-controlled human intervention trial [40]. The results indicated that B420 consumption modulated the gut microbiota—both alone, as well as in synbiotic product containing prebiotic fiber with probiotic—of an overweight study population towards the composition associated with a lean phenotype [40]. B420 alone was shown to increase the relative levels of beneficial microbes, such as *Lactobacillus* spp. and *Akkermansia* spp. [40]. Furthermore, *Bifidobacterium* spp. was

positively correlated with lean body mass, a finding that is in accordance with a previous publication reporting significantly less fat mass after B420 consumption compared to placebo [20]. A synbiotic product consisting of B420 together with polydextrose (PDX, a water-soluble branched oligomer of glucose and sorbitol classified as dietary fiber) administered in a human clinical trial [20] showed an increase of the relative proportion of *Akkermansia* spp., Christensenellaceae, and *Methanobrevibacter* spp. in the human fecal microbiota, while the prevalence of *Paraprevotella* spp. was reduced [40]. Christensenellaceae positively correlated with the fecal branched chain fatty acids (BCFAs) [40], which in previous studies have been indicated to inhibit de novo lipogenesis, thus potentially affecting lipid and glucose metabolism in human adipocytes [41]. Further, in the synbiotic group where an increase in the prevalence of Christensenellaceae was seen, this negatively correlated with energy intake, waist-hip-ratio at baseline, waist-area body fat and cholesterol markers [20,40].

2.2. Influence of B420 on Weight Management

Amar and colleagues demonstrated in mice that B420 supplementation was able to attenuate fat mass gain in obese and diabetic mice, concomitantly decreasing the translocation of commensal intestinal bacteria into blood and adipose tissue increased by high fat diet (HFD)-induced diabetes [25]. Moreover, in another mouse study, B420 was shown to reduce fat mass accumulation (1.89 g compared to placebo after a six week intervention, $p = 0.02$) in HFD-induced diabetic mice, when supplemented for six weeks as a single strain [23].

In line with these results, it has also been shown in a post hoc factorial analysis of a randomized clinical study that B420 supplementation resulted in significantly less total body fat mass (−4%, $p = 0.002$ vs. non-B420 containing groups, per protocol (PP) population) and waist circumference (−2.4%, $p = 0.004$ vs. non-B420 containing groups, PP population). In addition, the effect seemed to be concentrated in the fat localized in the central region of the body and thus seen as favorable changes in trunk fat mass ($p = 0.0002$) and android fat mass ($p = 0.004$) in the PP population when compared to groups not consuming B420 [20]. Combining B420 with other probiotics, or prebiotics—as synbiotic combination products—offers further possibilities regarding metabolic health. In the previously mentioned clinical trial, a synbiotic consisting of B420 with PDX was able to control body fat mass accumulation after a six month intervention with an average difference of 1.4 kg in total body fat mass ($p = 0.02$) between the synbiotic group and placebo group in the PP population [20]. The difference in body fat mass was most evident in the trunk region where there was 6.7% less fat mass ($p = 0.008$) and a 2.7% (2.6 cm) smaller waist circumference ($p = 0.047$) in the synbiotic group compared to the placebo group at the end of the intervention [20]. As trunk fat accumulation is associated with ectopic fat accumulation, controlling this inner organ fat is crucial for metabolic health. In the context of potentially synbiotic effects, an in vitro study showed that B420 was only weakly able to utilize PDX for growth compared to other complex oligosaccharides, xylo-oligosaccharide (XOS), fructo-oligosaccharide (FOS), or galacto-oligosaccharide (GOS) [42]. This enables sustained fermentation of PDX throughout the colon, but also indicates that intestinal survival of B420 is likely not dependent on the presence of PDX as a substrate.

Furthermore, in the human intervention trial, B420 significantly reduced energy intake by approximately 210 kcal/day ($p = 0.037$) compared to the non-B420 containing group [20]. This finding was supported by the earlier in vitro findings, in which the expression of satiety marker peptide YY (PYY) was shown to be increased by B420 [43]. The role of the intestinal microbiota in host appetite and food intake has been suggested to be conveyed through both regulation of eating-related behavior, possibly via the microbiota gut-brain axis [44], as well as via directly acting on molecules regulating appetite and satiety [45]. Therefore, some of the effects on metabolic health observed in clinical trials with B420 might be a result of changes in satiety and appetite hormone levels affecting food intake. However, this hypothesis requires further validation.

2.3. Influence of B420 on Glycemia, Lipidemia, Insulin Sensitivity, and Cardiovascular Disease Risk

Chronic overnutrition eventually leads to hyperlipidemia and hyperglycemia, which in turn, can cause insulin resistance via activation of stress-response and inflammatory signaling pathways [46]. Hormonal regulation of carbohydrate and other energy-rich nutrient metabolism is closely intertwined, and as previously stated, gut microbiota composition can affect the energy harvesting capacity by, e.g., modulating the number and affinity of transporter receptors [29]. Binding of insulin to its receptors normally results in the translocation of glucose transporter 4 (GLUT4) to the plasma membrane and glucose absorption (Figure 1), but also to upregulation of lipogenic activity [47,48]. The biochemical abnormalities in the diabetic state trace back to reduced entry of glucose, as well as to overaccumulation of lipids [49].

In the resting state of the liporegulatory system, when caloric intake is equal to expenditure, lean tissues contain little or no unmetabolized lipids. Positive energy balance promotes an increased mass of adipose tissue by hypertrophy (increased adipocyte cell size) and hyperplasia (increased adipocyte cell number), to buffer the effects of surplus energy intake on lean tissues. Hyperplasia promotes the secretion of antiobesity hormones such as leptin. Leptin was initially identified as a satiety hormone involved in energy balance by regulating fat storage, but it has also been shown to be involved in immune responses via modulating cytokine Th1/Th2 balance and promoting inflammatory response [50]. Hyperplasia enhances lean tissue oxidation of surplus lipids through downregulation of lipogenic enzymes [51,52]. Moreover, these events activate fatty acid beta-oxidation, further increasing the oxidation of surplus fatty acids [53]. Adipocytes have likely developed to buffer plasma fatty acid concentration by storing large quantities of triacylglycerols as non-specialist cells [52]. However, in insulin resistance, delayed hyperinsulinemia increases ectopic fat accumulation, closing the vicious cycle of ectopic fat accumulation and impaired glucose tolerance [54]. Leptin is involved in modulation of inflammation through the T cell compartment, forming a link between excessive fat accumulation and various inflammatory states [50].

Since the associations between gut microbiota composition and metabolic health have been observed, probiotics have been suggested as a potential therapeutic tool to improve insulin sensitivity. Promising indications have been obtained from a study by Vrieze et al. (2012), in which a fecal microbiota transplant from a lean donor improved insulin sensitivity in men with metabolic syndrome, indicating the ability of gut microbiota modification to restore impaired glucose intolerance [55].

The ability of B420 to support weight management has been shown to be associated with an attenuation in the progression of metabolic health disorders in dietary mouse models of diabetes and obesity [23,25]. In a diabetes mouse model study, B420 normalized the insulin sensitivity and fasting hyperinsulinemia with the fasting blood glucose of the B420 + HFD group (6.9 mM) being at the same level as that of a normal chow diet group (6.7 mM) and significantly lower than in the HFD alone group (8.2 mM, $p < 0.05$), and similar results were obtained from the glucose turnover rate. Additionally, positive effects were seen in tissue inflammation as the expression of major proinflammatory cytokines, interleukin (IL)-6, IL-1β, and plasminogen activator inhibitor (PAI)-1, was reduced ($p < 0.05$) in mesenteric adipose tissue [25].

Further, the gut microbiota also seems to play a role in the progression of cardiovascular diseases. So far, a direct association between the severity of myocardial infarction and gut microbiota composition has been shown in mice [56]. Moreover, in a recent study by Danilo et al. (2017), B420 mitigated the pathological impact of myocardial infarction in a mouse model [57]. In this study, myocardial infarction was induced in mice by an ischemia/reperfusion method after pre-treatment with either placebo, B420, or *Lactobacillus salivarius* Ls-33 [57]. Pretreatment with B420 for four weeks attenuated the cardiac injury by reducing significantly ($p < 0.05$) the infarct size and area when compared to saline-treated mice and a quenched inflammatory transcriptional profile resulting in lower levels of inflammatory markers such as IL-6 in the infarct area [57]. The observed associations can be mediated by the microbial metabolites interacting with cell surface receptors such as kinases containing ion channels located on the heart cell surface [58]. Lam et al. (2016) suggested that the observed beneficial

effects of probiotics on myocardial infarction are due to low molecular weight metabolites, such as phenylalanine, tryptophan, and tyrosine metabolites, produced by the intestinal microbiota, which affect protein kinases and potassium channels in signal transduction pathways [58]. Therefore, clinical studies to further elaborate the ability of B420 to alleviate myocardial infarction are warranted. Moving forward, it would be interesting to study how B420 functions to direct the metabolism of the microbes in a complex environment such as the gastrointestinal tract.

A benefit for combining B420 with antidiabetic drugs has been proposed based on a mouse study [26]. Whereas a low dose of metformin alone reduced plasma insulin concentration, the probiotic showed a similar effect to antidiabetic drug by lowering plasma glucose levels (B420 alone: 9.77 mmol/L, metformin with B420: 10.3 mmol/L, control: 10.8 mmol/L, metformin: 11.4 mmol/L, $p = 0.02$) and improving glucose regulation (B420 alone: AUC 2140 mmol/L*min, metformin + B420: 2160 mmol/L*min, control: 2340 mmol/L*min, metformin: 2660 mmol/L*min, $p = 0.002$ [26].

Furthermore, in the same study, a synbiotic product including B420 with PDX showed benefits for glycemic response and fasting plasma glucose in mice. Fasting glucose was not affected by sitagliptin, a medication used to treat diabetes mellitus type 2, whereas B420 both alone and in combination with PDX induced a statistically significant decrease [26]. Similarly, Garidou et al. obtained interesting results in a mouse model in which a type 2 diabetes resembling state with glucose intolerance, insulin resistance, and dysbiosis in the gut microbiota was induced by HFD [59]. The HFD-induced microbiota dysbiosis caused a decrease in the numbers of IL-17/ROR$_\gamma$t T cells and Treg cells in the small intestinal lamina propria, but when the HFD-fed mice were given B420 with PDX as a synbiotic treatment, the T cell numbers were similar to the level of the normal chow-fed mice [59]. Further, the fasting glycemia of synbiotic and HFD-fed mice was lower (4.9 mM) than that of mice receiving only HFD (7.5 mM), and similar to that of conventional normal chow-fed mice [59]. Furthermore, in a placebo-controlled, double-blind, randomized crossover trial, in which B420 was administered as a probiotic combination including *Lactobacillus acidophilus* 74-2 for a five week intervention period, the combination affected positively plasma lipid profile in healthy adults [60]. The concentration of triacylglycerols decreased significantly by 11.6% ($p = 0.045$) during the probiotic period, but no changes were detected in cholesterol levels, which might be due to the short intervention period [19].

2.4. Metabolic Endotoxemia and Chronic Low-Grade Inflammation in Gut Dysbiosis

Gut dysbiosis refers to a state of microbial imbalance caused by perturbations in the structure or functions of the microbial communities [61,62]. Microbiota disruption can result in the loss of beneficial microbes, reduced diversity or pathobiont expansion, with normally dominating species becoming underrepresented and outcompeted by atypical organisms.

An inflammatory response is a complex self-limiting process coordinated by vasoactive amines, adhesion molecules, lipid-derived eicosanoids, cytokines, and chemokines. Inflammation is fundamentally a protective mechanism. However, when the self-limiting nature of this process is inappropriately regulated, it is transformed into a detrimental, chronic state of inflammation, often referred to as chronic low-grade inflammation, which multiple studies have indicated to play a crucial role in metabolic disorders [63].

The dysbiosis paradigm recognizes the interrelations of gut microbiota and metabolic health in more detail and is based on the idea that gut microbiota composition can affect intestinal barrier function and thus regulate the translocation of inflammatory gut microbes and their components, which then cause tissue inflammation by affecting immunomodulatory metabolic pathways [64]. Obesity has been shown to be associated with increased gut permeability both in animal [65], as well as in human studies [66].

Amar and colleagues (2011) demonstrated in mice that translocation of commensal intestinal bacteria into blood and adipose tissue is increased during the onset of HFD-induced diabetes [25]. Moreover, the translocation results in low-grade bacteremia, and the presence of viable bacteria in the blood is further associated with CD14, a lipopolysaccharide binding protein, functioning as the

endotoxin receptor, and Nod1, a receptor recognizing bacterial molecules and inducing immune responses, and is regulated by adipokine leptin. A one month treatment with B420 reduced the mucosal adherence of *Escherichia coli* and bacterial translocation of Enterobacteriaceae into adipose tissue, thus reversing the bacteremia [25]. Furthermore, the expression of the major pro-inflammatory cytokines—TNF-α, IL-1β, and IL-6, and coagulation regulator PAI-1—was reduced in mesenteric adipose tissue, liver, and muscle by B420 and associated with positive changes in insulin sensitivity, as described in more detail earlier in this review [25].

Metabolic endotoxemia refers to the hypothesis in which microbes [25], or microbial fragments, such as lipopolysaccharide LPS [67], peptidoglycan [68], and flagellin [69], enter the bloodstream from the gut and end up in different tissues, causing exaggerated lipolysis and low-grade inflammation. In an in vivo study, treatment with B420 decreased bacterial adherence to the intestinal mucus of mice [23]. Furthermore, in vitro findings from cell culture studies indicated the superiority of B420 among the screened strains in enhancing epithelial integrity in an intestinal epithelial cell model [21,22].

Later mice studies verified that similar results could be obtained in vivo, and B420 was shown to reduce epithelial translocation of *E. coli*, as well as to lower the circulating LPS levels in two separate study settings [23,25]. Once in circulation, LPS binds to LPS binding protein (LBP), activating the CD14 receptor and further the toll-like receptor 4 (TLR4) [70]. As TLR4 is connected to insulin metabolism via cytokine signaling, activation of TLR4 links LPS to insulin resistance presumably by altering insulin receptor signaling in the presence of inflammatory cytokines [71], as explained in more detail in Section 2.5.2. and illustrated in Figure 1. In addition, the interrelations of cellular signaling pathways, cytokines related to inflammatory responses, and insulin metabolism are illustrated in Figure 1.

In a clinical study, B420 appeared to keep the levels of circulating zonulin, a potential marker of intestinal permeability, consistently lower throughout the study compared to groups without B420 [20]. Furthermore, changes in inflammation marker high sensitivity C-reactive protein (hsCRP) were significantly correlated with the changes in zonulin, although they did not reach statistical significance as such [20]. The clinical results support the earlier preclinical findings that B420 improves epithelial barrier function.

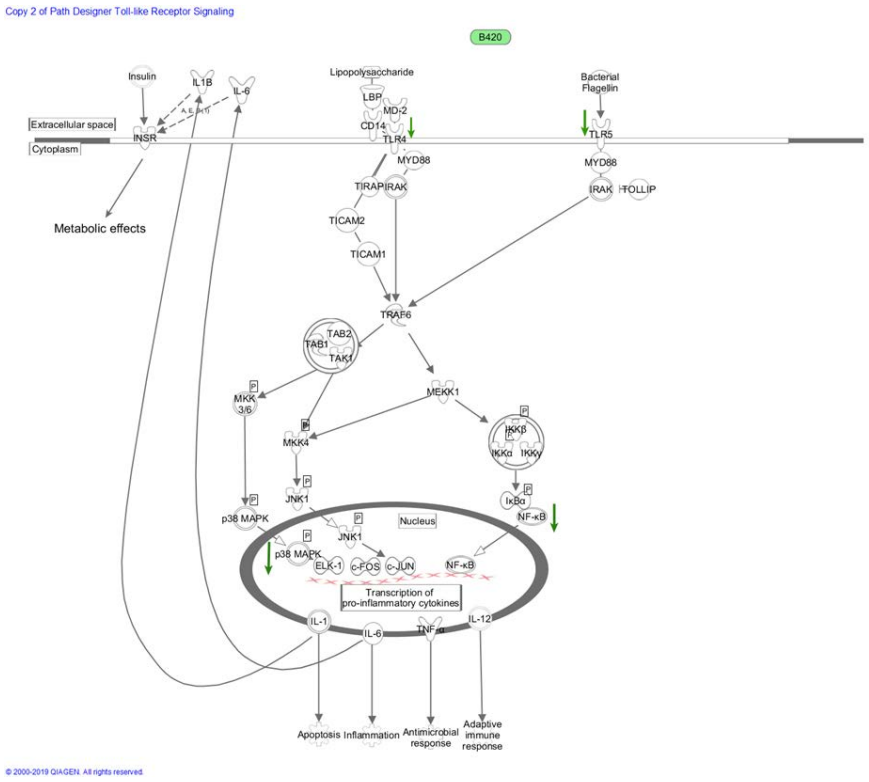

Figure 1. Schematic proposal of how B420 and/or its metabolites presumably affect cellular signaling via downregulation of TLR pathways, indicated as light green arrows in the figure, as well as the inflammatory IKKα-NF-κB pathways or p38 MAPK-ELK-1 pathways. B420 has been shown to enhance epithelial integrity in vitro [22] and to decrease the levels of circulating LPS in mice [27]. LPS triggers inflammation through upregulation of IKKα-NF-κB pathways and p38 MAPK-ELK-1 pathways [72], indicated as dark green arrows in the figure. Downregulation of these pathways by B420 reduces the transcription of pro-inflammatory cytokines in the nucleus. As proinflammatory cytokines are excreted out of the cell, and affect insulin receptor activity, this cascade can serve as one mechanistic route via which B420 exerts its metabolic effects through affecting epithelial integrity and cytokine levels. LPB = lipopolysaccharide binding protein; MD-2 = lymphocyte antigen 96, a protein associated with toll-like receptor 4; CD14 = cluster of differentiation 14; TLR4 = toll-like receptor 4; MYD88 = myeloid differentiation primary response protein 88; TIRAP = toll-interleukin 1 receptor (TIR) domain containing adaptor protein, IRAK = interleukin-1 receptor associated kinases; TICAM2 = toll-like receptor adaptor molecule 2; TICAM1 = toll-like receptor adaptor molecule 1; TRAP6 = thrombin receptor activator peptide 6; TAB2 = TAK1-binding protein 2; TAB1 = TAK1-binding protein 1; TAK1 = TGF-β activated kinase 1; MKK3/5 = mitogen-activated protein kinase kinase 3/5; p38 MAPK = p38 mitogen-activated protein kinase; MKK4 = mitogen-activated protein kinase kinase 4; JNK1 c-Jun N-terminal kinase 1; ELK-1 = ETS-like gene 1 (coding for ETS like protein Elk-1); c-FOS = Fos proto-oncogene, which is an AP-1 transcription factor subunit; c-JUN = Jun proto-oncogene, AP-1 transcription factor subunit; NF-κB = nuclear factor kappa B; TNF-α = tumor necrosis factor alpha; IL-12 = interleukin 12; IL-6 = interleukin 6; IL-1 = interleukin 1; MEKK1 = mitogen-activated protein kinase kinase kinase 1; IKKβ = I kappa B kinase beta; IKKα = I kappa B kinase alpha; IKKγ = I kappa B kinase gamma; TLR5 = toll-like receptor 5; TOLLIP = toll interacting protein; IL1β = interleukin 1β; INSR = insulin receptor. The schematic networks were generated through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

2.5. Immunomodulatory Pathways in Metabolic Endotoxemia

The mechanism by which the microbes or their fragments translocate from the gut in metabolic endotoxemia is unclear, but it is thought that the integrity of the epithelial layer in the intestinal

wall is compromised and the permeability is increased, which leads to translocation of luminal components [25,73,74]. It has been noted that, for example, dietary or antibiotic-induced modification of gut microbiota might lead to a reduction of inflammation and intestinal permeability [73,74]. The consumption of probiotics might also have similar effects, and B420 has been shown to improve epithelial barrier function in cell culture [21,22] and to reduce the epithelial translocation of *E. coli*, as well as circulating LPS levels in mice [23,25]. It is likely that this effect is induced also by other mechanisms than solely the modification of gut microbiota composition.

Immunomodulatory pathways refer to linked signaling pathways that are responsible for communicating signals by various agents and particles to different parts of the body to induce required responses. The connection between diet and intestinal permeability has been commonly accepted, and for example, the effect of dietary carbohydrate composition on postprandial hyperglycemia and postprandial insulin response is well established [75]. However, it is far more controversial how the signaling pathways serve as mechanistic linkages between diet, gut microbiota, and metabolic health delivering these effects. Lately, the ability of food-like components to alter postprandial and fasting state metabolism through modulation of various signaling pathways has been proposed. For example, the AMPK (adenosine monophosphate activated protein kinase)-SIRT1 (silent information regulator T1) cascade serves as an indicator of cellular energy status and is thus an important regulator of carbohydrate and fat metabolism [76]. As B420 has shown promising effects in many animal and in vitro trials related to metabolic health, modulation of these signaling pathways by B420 can certainly be considered as a potential mechanism mediating the observed effects and is worth further investigation (summarized in Figure 1).

2.5.1. Cyclooxygenase and Nitric Oxide Synthase Pathways

Cyclooxygenases (COX) are enzymes functioning in the rate-limiting step of the arachidonic acid cascade to form eicosanoids, thromboxanes, and prostaglandins, which can mediate vasoconstriction or inflammatory functions depending on the eicosanoid, receptor type, and distribution. In eukaryotic cells, two COX isoforms exist: COX-1, which is involved in the maintenance of the physiological functions in a constitutive way, whereas COX-2 as an inducible enzyme mediates mitogenic and inflammatory responses, even though continuous discussion exists about the exact roles of the two isoforms [77]. COX-2 is generally present in low levels in mammalian tissues, unless induced by one of many types of stimuli such as growth factors and cytokines [78].

Arachidonic acid is released from membrane phospholipids through phospholipase A_2 cleavage and can be metabolized through the COX pathway into prostaglandins and thromboxane A_2, or by the lipoxygenase pathway to hydroxy- and hydroperoxy-eicosatetraenoic acids and leukotrienes [79]. Inhibition of COX, lipoxygenase and phospholipase A_2 by for example plant secondary metabolites has been shown in many studies and results in lower circulating levels of these inflammatory eicosanoids in vitro [80], but the idea of probiotics inhibiting these enzymes is rather recent. In an animal study, treatment with a strain of *B. lactis* was able to suppress COX-2 expression and colonic TNF-α production in a trinitrobenzene-sulfonic acid-induced model of rat colitis [81]. Furthermore, in a rat model, B420 supplementation protected from an NSAID-induced increase in gastric permeability [27].

B420 has been shown to affect the COX pathway by producing metabolites that have been observed to upregulate COX-1 in an undifferentiated and differentiated human intestinal epithelial cell model, Caco-2, and concomitantly, downregulate the expression of COX-2 [22,82]. This function is similar to and has previously been elicited by butyrate and propionate, two well-known beneficial microbial metabolites [82]. This effect seems to be a species- and strain-dependent phenomenon, as several other bifidobacteria or lactobacilli share this COX gene regulating effect elicited by B420 [22,82]. In metastatic gastric adenocarcinoma cells, on the other hand, B420 was not able to regulate either COX-1 or COX-2 [83], which indicates that the regulation is cell type-dependent. B420 metabolites were also able to counteract the tight junction integrity-decreasing effect of *E. coli* O157:H7, which has an

opposite, COX-2 upregulating and COX-1 downregulating effect [22]. However, the role of eicosanoids in the barrier regulation of the intestinal epithelial cell model by B420 is currently unknown.

The nitric oxide synthase (NOS) pathway is important in the maintenance of bodily functions; endothelial nitric oxide synthase (eNOS) has an important role in maintaining blood pressure homeostasis and vascular integrity, while inducible nitric oxide synthase (iNOS) invokes an inflammatory process [84]. During the past decade, the significance of sustained high NO production by iNOS in intestinal inflammation and gastrointestinal immunopathology, such as chronic inflammatory bowel disease, has become evident [85,86]. The gut microbiota has been shown to regulate circulating amounts of iNOS via microglia activation [87]. Probiotic strains from both genera *Bifidobacterium* and *Lactobacillus* have been shown to possess eNOS and iNOS inhibition, respectively [88,89]. In a study by Putaala et al. (2010), intact bacterial cells of B420 were shown to induce the expression of iNOS via activation of transforming growth factor beta-activated kinase 1 (TAK1) and activator protein 1 (AP1), whereas the cell-free supernatant induced AP1 and inhibited TAK1 [90], indicating differential regulation by cells and bacterial metabolites. This is probably very important since bacterial cells should remain in the lumen of the healthy gut. In animal study designs, *B. lactis* treatment was able to reduce iNOS synthase expression and colonic TNF-α production in a trinitrobenzene-sulfonic acid-induced model of rat colitis [91]. More recently, a probiotic cocktail containing *B. lactis* (strains not reported) among three other probiotics (*L. acidophilus*, *Lactobacillus plantarum*, and *Bifidobacterium breve*) was shown to promote recovery from acute colitis via inhibition of iNOS, as well as the nuclear factor (NF)-κB pathway [86].

2.5.2. NF-κB and MAPK Pathways

Metabolic endotoxemia is associated with altered cytokine balance in serum favoring proinflammatory cytokine production (such as IL-6, IL-8, TNF-α) over that of anti-inflammatory cytokines (such as IL-10, TGF-β, IL-4). Interfering with this finetuned balance may induce overly active proinflammatory cytokine production in all tissues [46]. Obesity-induced inflammation is partly due to toll-like receptor (TLR) activation [92]. TLRs are innate immune receptors on the cell surface or in the intracellular membranes that recognize various microbe-derived molecules, such as bacterial lipoteichoic acid (TLR2), LPS (TLR4), flagellin (TLR5), and CpG DNA (TLR9), among many others. The activation of TLRs via different adaptor proteins leads to the activation of complex signaling pathways that result in the activation of cytokine gene transcription to induce a proper innate immune response to fight the pathogen. The main signaling pathways coordinating TLR signaling are interferon regulatory factors (IRF), the mitogen-activated protein kinase (MAPK) pathway, and the NF-κB pathway, the latter functioning as the central valve of many chemokines, adhesion molecules, growth factors, acute-phase proteins, cell proliferation, iNOS, invasion, migration, and immune receptors, making it a potent anti-inflammatory target [93].

Not surprisingly, many health-promoting food components such as polyphenols have been shown to exert their functions via inhibition of the NF-κB pathway [94], and recently, the ability of probiotics or gut microbiota metabolites to regulate the NF-κB pathway has gained interest. Overall, there is emerging evidence that probiotics are able to modulate innate pathogen sensing signaling pathways, as reviewed recently by Llewellyn and Foey [95]. However, to date, the data are rather sparse as the responses may be either inhibiting or activating, depending on the probiotic strain, cell/tissue type, experimental setup, and which components of the signaling pathways were analyzed. In an in vitro study by Putaala et al. (2010), the cell-free metabolites of B420 were shown to have to some extent opposite effects on known NF-κB pathway regulator gene expressions than enterohaemorrhagic *E. coli* O157:H7 (EHEC) [90], a pathogenic bacterium known to induce inflammation in intestinal epithelial cells in vitro [22]. Thus, these results can be interpreted to indicate that downregulation of NF-κB pathway presents one possible route through which B420 could affect TLR signaling.

The mitogen-activated protein kinases (MAPK) are a chain of sequentially activated protein kinases that regulate many different cell processes [96]. The MAPK family consists of Ser/Thr kinases

including at least extracellular signal-related kinases (ERK)1/2, c-Jun amino-terminal kinases (JNK) 1/2/3, p38-MAP kinase, and ERK5 kinases, of which the JNK and p38 cascades are most involved in inflammation [97]. Cell-free metabolites of B420 were shown to downregulate p38 and ERK in an in vitro study assessing the transcriptional response of human intestinal epithelial cells to various probiotics [90], indicating the ability to influence host cytokine levels via affecting the complex signaling pathways that coordinate cytokine production.

One interesting target for probiotic modulation is TLR4 signaling, which is central in metabolic endotoxemia [98] and linked to obesity, as TLR4-deficient mice are resistant to HFD-triggered obesity [99]. Several lactobacilli strains have been shown to regulate TLR4 signaling negatively such as *Lactobacillus casei* OLL2768 via inhibiting the NF-κB and p38 pathways and upregulating negative regulators Tollip and Bcl-3 in bovine intestinal epithelial cells stimulated with heat-killed enterotoxigenic *E. coli* (ETEC) [100] and *Lactobacillus amylovorus* DSM 16698^{T} by suppressing the ETEC-induced activation in the human intestinal Caco-2/TC7 cell line [101]. Similarly, B420, as well as its cell-free metabolites downregulated TLR4 gene expression in intestinal epithelial cells [90]. However, in a human trial, daily consumption of B420 did not lower IL-6 cytokine levels in overweight and obese adults who had normal range levels at baseline [20], indicating that further research is needed to elaborate these mechanisms in more detail with B420. Intriguingly, mice lacking TLR5, the receptor for bacterial flagellin, developed metabolic syndrome, and their gut microbiota was altered [69]. As B420 decreased TLR5 gene expression in intestinal epithelial cells [90], it would be of great interest to study whether downregulation of TLR signaling and subsequent suppression of inflammation comprise one of the mechanisms by which beneficial microbes may reduce metabolic syndrome and obesity. The proposed mechanistic route through which B420 could affect metabolic health via TLR4 signaling is summarized in Figure 1.

In the search for effective and safe solutions for the worldwide obesity epidemic, B420 has arisen to provide warranted benefits without safety concerns. The ability of B420 to adhere to intestinal mucosa is the basis of all observed beneficial effects. Considering the results above, it seems possible that B420—both alone and as part of synbiotic products—may have a positive modulatory effect on gut microbiota composition by increasing the relative abundance of other bacterial genera with known beneficial effects in the gut. Further, it seems that B420 is able to affect gut microbiota composition in an anti-obesogenic manner by increasing the prevalence of lean phenotype microbes such as *Akkermansia muciniphila*.

3. Conclusions

All in all, it seems evident that B420 has significant beneficial effects on weight management and metabolic health mediated by a complex signaling pathway network yet to be fully understood by current research. The research aiming to understand the impact of B420 on the mechanisms both inside the gut, including mucosa and sub-mucosa (epithelial barrier function), as well as the gut immune system, and at a systemic level has been well initiated. In the future, it will be extremely intriguing to dig deeper into the world of systemic metabolism utilizing machine learning approaches to filter through these complex pathways and find meaningful correlations. Bacterial metabolites have been suggested to be in part responsible for the observed effects of probiotics in general. Future research should also focus on examining the health benefits these bacterial metabolites can have in human.

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