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# Association of Probiotics with Gut Flora in Early Life and its Effects on Obesity in Mice

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Mustafa Alsharafani

**Some results of this work have been published:**

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## LIST OF ABBREVIATIONS

| Abbreviations | Definitions |
|---|---|
| ACC | Acetyl-CoA carboxylase |
| AL | Acetylglucosamine - Lactose medium |
| AMPK | Adenosine monophosphate activated protein kinase |
| ANOVA | Analysis of variance |
| B | *Bifidobacteria* |
| BBM | Bifidobacterium selective agar medium |
| bp | Base pair |
| BS | Bifidobacterium selective agar medium |
| CFU | Colony forming unit |
| CPT-1 | Palmitoyl transferase-1 |
| EDTA | Ethylenediaminetetraacetic acid |
| F6PPK | Fructose-6-phosphate phosphoketolase |
| FELASA | Federation of Laboratory Animal Science Associations |
| FI | Food intake |
| FOS | Fructoologosaccharides |
| G | Glucose |
| GIT | Gastro-intestinal tract |
| HFD | high-fat diet |
| IVC | individually ventilated cages |
| L | *Lactobacillus* |
| LCL | Liver Cysteine Lactose medium |
| LSD | Least significant differences test |
| MALDI TOF | matrix-assisted laser desorption ionization-time of flight mass |
| MRS | de Man, Rogosa and Sharpe |
| NADH | Nicotinamide Adenine Dinucleotid Hydrogen |
| NASH | Non-alcoholic steatohepatitis |
| $n_d$ | number of days |
| $n_m$ | number of mice per cage |

| | |
|---|---|
| Olig | oligofructose |
| PFA | Paraformaldehyde |
| RCB | Cephalothin and blood medium |
| SPF | specific pathogen-free |
| TOS | Transoligosaccharide propionate agar medium |
| TPCY | Tomato Casein Peptone Yeast agar medium |
| $w_0$ | weight (g) of the food provided |
| $w_{ed}$ | weight (g) of the food remaining |
| YE | yeast extract |

# 1 Introduction

## 2. Bifidobacteria

The disequilibrium between energy intake and expenditure causes obesity (Woods *et al.* 1998). The major cause of obesity is a positive energy balance resulting from increased dietary energy intake and decreased energy output. Recent studies have showed that microbiota can contribute to the development of obesity (Cani *et al.* 2007). Non-alcoholic fatty liver disease (NAFLD) comprises a series of liver injury associated with liver cell fat accumulation in the absence of significant alcohol consumption. Non-alcoholic steatohepatitis (NASH) an often silent liver disease is a part of the NAFLD and is characterized by the presence of excess fat in the liver accompanied by variable liver cell inflammation or fibrosis. NASH is often associated with obesity, becoming increasingly prevalent both in adults and children (Chalasani *et al.* 2012; Kleiner *et al.* 2005). Ley *et al.* (2005) indicated, in a rodent experiment that the obesity could be connected to an altered gut microbiota. The gut microbiota contributes to the host response towards nutrients (Qin *et al.* 2010; Diamant *et al.* 2011). During their first year of life, normal weight children exhibited a fecal microbiota richer in bifidobacteria but poorer in *Staphylococcus aureus* than children who were subsequently overweight (Kalliomäki *et al.* 2008). Genus *Bifidobacterium* (*B.*) are broadly characterized as Gram-positive, non-motile, catalase-negative and obligate anaerobic bacteria. Bifidobacteria are capable of utilizing a variety of complex carbohydrates which are indigestible by the host during transit through the gastro-intestinal tract (GIT). *Bifidobacterium* species are common inhabitants of the human GIT of human, different animals and sewage (Pokusaeva *et al.* 2011; Biavati *et al.* 2006). In human some bifidobacteria can be opportunistically pathogenic, such as *B. dentium*, others are commensal or health-promoting, also classified as probiotic microorganism like *B. bifidum* and *B. breve* (Vitali *et al.* 2007; Bhadoria *et al.* 2011; Kumar *et al.* 2012). Bifidobacteria have many beneficial effects including protection against pathogens as it plays a role in the normal development of the immune system and positive nutritional effect on the intestinal cells and the host (Venema *et al.* 2003).

In a previous study, *B. longum* showed a more significant effect in lowering serum total cholesterol than a mixed culture of *Streptococcus thermophilus* and *Lactobacillus*

*delbrueckii* subps. *bulgaricus* both in rats and humans. These results suggested that some specific strains of bifidobacteria linked to lipid metabolism and body weight may be potentially therapeutic when applied for managing obesity (An *et al.* 2011). Attempts have been made to increase the number of bifidobacteria in the intestinal tract by supplying specific bifidobacteria strains and foodstuffs that stimulate the growth of *Bifidobacteria* (Kitajima *et al.* 1997; Lee *et al.* 1999). It has been proposed that alterations in the composition of the gut microbiota known as dysbiosis in the triglyceride accumulation and hypercholesterolemia, and that participated in obesity (Neyrinck *et al.* 2012). The development of obesity and type 2 diabetes following feeding a high-fat diet was characterized by specific changes of the bacterial populations which were predominant in the gut microbiota. Yin *et al.* (2010) concluded that the response of energy metabolism to bifidobacteria was strain-dependent. Therefore, different strains of bifidobacteria might lead to different fat distributions.

The overall aim of this study was to evaluate the anti-obesity activity of Bifidobacteria strains grown in skimmed milk media on young mice fed a high-fat diet. This is achieved by:

- Isolation of predominant *Bifidobacterium* species from stools of breastfed infants which have been identified as a probiotic.
- Improved the growth of *B. breve* and *B. longum* in skimmed milk media supplemented with yeast extract, glucose and oligofructose on different levels that increase count number after conservation.
- Increasing the amount of bifidobacteria in the host intestine.
- Evaluation of the effect of bifidobacteria strains on weight gain and liver lipid accumulation as well as serum triglycerides on young mice fed a high-fat diet.

## Early studies of the Bifidobacterium genus

Henry Tissier isolated bifidobacteria based on his observation of microbiota in the stools of breast-fed infants. Tissier named his find *Bacillus bifidus communis* which is now known as bifidobacteria (Tissier 1899; Tissier 1900). Later, Tissier's isolates were named *Lactobacillus bifidus* following Winslow's suggestion that they belonged to the family of Lactobacillaceae (Holland 1920). The Danish microbiologist (Orla-Jensen

1924) differentiated the genus bifidobacterium into a separate taxon and showed similarities to the genus Lactobacillus that he listed in the seventh edition of Bergey's *Manual of Determinative Bacteriology* (Breed *et al.* 1957). Although the cell wall murin structure of the bifidobacteria is more similar to Lactobacillaceae than to Actinomycetaceae, *Bifidobacterium* species were included in the family Actinomycetaceae of the order Actinomycetales of the Actinomycetes (Kandler *et al.* 1974). Further studies have recognized the presence of several multiple biotypes of Bifidobacterium, and Dehnart suggested a scheme for the differentiation of these bacteria on the basis of their ability of carbohydrate fermentation (Biavati *et al.* 2000). (Reuter 1963) recognized *B. bifidum* and defined seven species of bifidobacteria. The key enzyme, fructose-6phosphate phosphoketolase of hexose pathway fermentation was characterized by Scardovi *et al.* (1965) and later by (Devries *et al.* 1967). Scardovi *et al.* (1970) started to use the DNA-DNA filter hybridization procedure to recognize DNA homology groups among the previously described strains and to recognize new isolates from different ecological niches. Rogosa (1974) classified the same name of the genus Bifidobacterium in the eighth edition of Bergey's *Manual of Determinative Bacteriology*. Some corrections were made to the classification after presentation of the electrophoresis of soluble cell proteins on polyacrylamide gel as a criterion for rearrangement of species' identification (Biavati *et al.* 1982). Stackebrandt *et al.* (1997) suggested based on 16S rRNA analysis, a novel hierarchy structure collection of the genus Bifidobacteria with the genus *Gardnerella* into the single family of Bifidobacteriaceae in the order of Bifidobacteriales.

The Bifidobacterium cells have been defined as irregular, non-capsule, non-motile, non-spore-forming, non-filamentous and Gram-stain posative. Bifidobacteria are anaerobic (lacking $H_2O_2$ detoxification systems) with an optimum growth temperature of 35–39°C. Bifidobacteria show negative reactions for indole fermentation, gelatin hydrolysis, catalase (with an exception for *B. indicum* and *B. asteroides* when grown in the presence of air) and oxidase, where neither release $CO_2$ from carbohydrate fermentation nor produce butyric or propionic acids (Baffoni *et al.* 2013; Biavati *et al.* 2006). At present, the genus of bifidobacterium includes 39 species (Pokusaeva *et al.* 2011; Okamoto *et al.* 2008).

Bifidobacterium cells appear in various morphological forms during isolation and culturing. The characteristic shape of bifidobacteria is a rod-shaped form with bifurcated Y- and V-forms that occur singly, in clumps or in many-celled chains. Coccoid forms can also be seen. The bulge often occurs at one end in the form of a cudgel or spatula and sometimes as a branch. Different forms can be found within one species depending on the culture conditions (Kojima *et al.* 1970). The concentration of calcium ions and sodium salts involved in the synthesis of the cellular wall results in an increase of branches (Glick *et al.* 1960). In 1992, Biavati *et al.* suggested that pleomorphism is apparently affected by a defective synthesis of the cell wall rather than because of degenerative processes and the observed phenomenon of 'phase variation'. The irregular shape is a result of the absence or low concentrations of *N*-acetylamino amino acids (alanine, aspartic acid, glutamic acid, and serine) or sugar (Biavati *et al.* 2006). The cell morphology is affected by different culture conditions and species-specific dimensions (Poupard *et al.* 1973). The colonies are opaque and the shape is mostly spherical (Biavati *et al.* 2006).

Bifidobacteria can grow in semi-synthetic media-supplemented factors that are used for lactobacilli (Poupard *et al.* 1973). Bifidobacteria are chemoorganotrophs, possessing a fermentative type of metabolism. Bifidobacteria show the ability to grow on ammonium salts as the sole nitrogen source (Mattarelli *et al.* 1999). Although there is a heterogeneity in the nutrient requirements of bifidobacteria species, riboflavin and calcium pantothenate are the main nutrients required for growth while the other water-soluble (B-complex) vitamins can be synthesized by several strains isolated from humans (Trovatelli *et al.* 1978). *Bifidobacterium pseudolongum* subsp. *globosum* shows auxotrophy for L-leucine amino acid for plasmid cloning requirement (Mattarelli *et al.* 1999). Bifidus factors are able to enhance the growth of bifidobacteria that can be classified into oligosaccharides like fructooligosaccharides and glycoproteins such as N-acetyl-D-glucoseamine or iron-binding proteins such as lactoferrin (Bezkorovainy *et al.* 1981); (Pokusaeva *et al.* 2011a). Bifidobacteria have efficient system enzymes for breaking down oligosaccharides and polysaccharides, especially fructofuranosidase (Perrin *et al.* 2001). Bifidobacteria's ability to simulate oligosaccharides and polysaccharides offers competition to the other microorganisms in the intestinal tract (Van der Meulen *et al.* 2004). The cell mass of *B. breve* 255 was enhanced in a modified

MRS media containing 1% (w/v) sodium thiosulphate. The yield biomass of *B. breve* 255 was optimized by 37.4°C, pH7.09, inoculum volume 1.97 (ml/100mL), inoculum age 58.58 hours, carbon content 41.74% and nitrogen content 46.23% (Meena *et al.* 2011).

More than 31 different cultural media were created for isolating and enumerating *Bifidobacterium* species from human, animal, environmental and food sources. Shigwedha *et al.* (2013) specified the popular media used for the enumeration of bifidobacteria from different sources. They classified the media into five different groups. The first group is a non-selective medium (such as MRS and Rogosa), a medium without antibiotics but with elective carbohydrate (Acetylglucosamine–Lactose [AL]) agar and Liver Cysteine Lactose (LCL) agar, a medium with antibiotics (such as Bifidobacterium Iodoacetate Medium (BIM-25) agar, reinforced Clostridial agar with Cephalothin and Blood (RCB) and Tomato Casein Peptone Yeast agar (TPCY with antibiotics), a medium with propionate (Propionate agar or Beerens agar), and a medium with elective substance and/or low pH (Tomato Casein Peptone Yeast agar [TCPY], Tomato Casein Peptone Yeast agar [TPCY] with azide, Tomato Casein Peptone Yeast agar [TPCY with sorbic acid, TTC-agar and x-Gal medium]. Combinations belonging to more than one group are also used—for instance, Bifidobacterium selective [BS] agar, Bifidobacterium selective medium [BBM] agar, Modified Rogosa agar, MPN-agar, MRS agar with LiCl and antibiotics [MRS-NN], Neomycin Paromomycin Lithium Nalidixic acid [NPLN] agar, Raffinose–Bifidobacterium [RB] agar and YN-6 agar). It can be concluded from the large variety of the types of media that there is no standard medium for the detection of bifidobacteria (Roy 2001).

Bifidobacteria break down sugars through a metabolic pathway (Figure 1) to acetate and lactate for energy production by a system called ‘bifid shunt’. The key enzyme fructose-6-phosphoketolase (EC 4.1.2.2) is considered to be a taxonomic marker for the family of Bifidobacteriaceae.

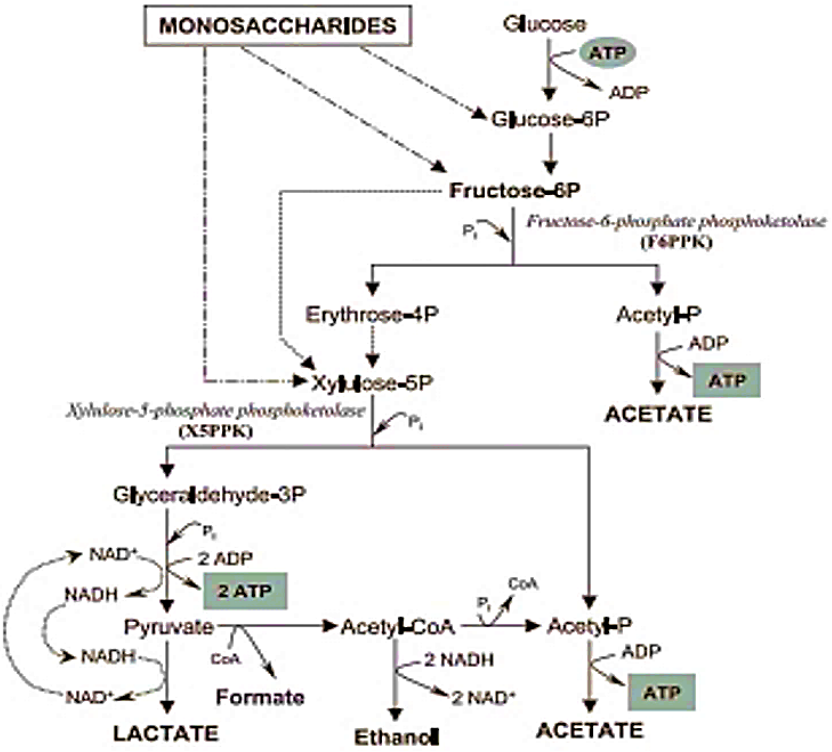


**Figure 1: Schematic representation of the main steps of the fructose-6-phosphate shunt in Bifidobacterium.**

Further enzymes are required to produce energy in the form of ATP from fermentation of carbohydrates (Reyes-Gavilan *et al.* 2005). From the fermented one-molecule glucose the bifid shunt pathway of Bifidobacteria produces 2.5 ATP mol more energy than the homofermentative group of lactic acid bacteria that produces 2 ATP mol, while heterofermentative lactic acid bacteria produces 1 mol of ATP. Bifidobactria can assimilate a wide variety of sugars, such as glucose, fructose, galactose, arabinose, xylose, ribose, sucrose, lactose, cellobiose, melibiose, gentobiose, maltose, isomaltose, raffinose and mannose. In addition, the carbohydrate-derived substrates *N*-acetylglucosamine and *N*-acetylgalactosamine, mucin and pectin could be assimilated (Pokusaeva *et al.* 2011; Reyes-Gavilan *et al.* 2005; Bezkorovainy *et al.* 1989).

The key enzyme (fructose-6-phosphate phosphoketolase F6PPK) cleavages hexose in the bifid shunt pathway to erythrose phosphate and acetyl phosphate leading to an increase in lactic acid and acetic acid in a theoretical ratio of 1:1.5 which may change according to the assimilated carbon source and the species type (Palframan *et al.* 2003). Xylulose phosphates are formed from erythrose and fructose phosphates

metabolite through the consecutive activity of transaldolase and transketolase increasing the ratio of acetic acid to lactic acid. The glyceraldehyde 3-phosphate generated enters the Embden-Meyerhoff-Parnas pathway (Ballongue 1998). Some of the pyruvate is converted to formic causing a reduction of acetate to ethanol (Lauer *et al.* 1976; Schramm *et al.* 1958).

Due to the lack of electron acceptors in anaerobic organisms, the organic substance is subjected to a series of oxidation and reduction reactions, and hydrogen peroxide is accumulated. The deficient scavenging enzymes in bifidobacteria such as superoxide dismutase, catalase, and peroxidases are present in aerobic organisms that cannot eliminate lesions in proteins and nucleic acids of oxygen toxicity (Imlay 2008). It has been proposed that the sensitivity of *Bifidobacterium* species to oxygen is due to the existence production system of $H_2O_2$ and the absence of detoxification systems of $H_2O_2$. The inhibition of an essential metabolic enzyme of bifidobacteria in the presence of oxygen may be due to the inactivation of pyruvate: Ferredoxin oxidoreductase (Pan *et al.* 2001). Shimamura *et al.* (1990) suggested that the activities of Nicotinamide Adenine Dinucleotid Hydrogen (NADH) oxidative enzyme are very low in *Bifidobacterium* species in comparison to the activities that are found in $O_2$-tolerant species. The increased activities of NADH peroxidase correlates with the increase in hydrogen peroxide decomposition (Talwalkar *et al.* 2004). Most of *Bifidobacterium spp.* grew successfully without inhibition in the presence of 5% $O_2$ but stopped growing in 10% $O_2$ while *B. boum* JCM1207 grew well in the presence of 20% $O_2$. The degree of tolerance to oxygen depends on the particular type of *Bifidobacterium* species and culture medium. The *Bifidobacterium spp*. can be classified into four groups; $O_2$-hypersensitive, $O_2$-sensitve, $O_2$-tolerant, and microaerophilic. The species belong to the $O_2$-tolerant and microaerophilic groups and are all isolates of nonhuman sources: sewage (*B. minimum*), bovine rumen (*B. boum* and *B. thermophilum*), and (*B. astroides* and *B. indicum*) from the honey-bee hindgut (Kawasaki *et al.* 2006).

Bifidobacteria belong to the phylum Actinobacteria, class Actinobacteria, order Bifidobacteriales, and family Bifidobacteriaceae (Garrity *et al.* 2007; Otieno 2011). Bifidobacteria are distinguished according to phylogenetic characteristics, belonging to the high G-C (42-67%) and usually higher than 55% of the content are different from the Lactobacillus genera. Consequently, lactic acid is one of the main fermentation products

of bifidobacteria; therefore, they were included in the group of lactic acid bacteria, which share the same features as that of the host habitat. Depending on a number of general fermentation product features, bifidobacteria and lactic acid bacteria are the most commonly used probiotic microogranisms in the food industry (Bhadoria *et al.* 2011). The efficacy of organic acid production of *Bifidobacterium* species toward pathogens is greater than the effects of lowering the pH value alone. When acetic and lactic acid have been used for pH value reduction, the growth inhibition of *Salmonella enterica Typhimurium* and *E. coli* was greater than hydrochloric acid indicating the involvement of other mechanisms (Makras *et al.* 2006).

The phenotypic identification of bifidobacteria depends on the morphological features and evidence for the presence of Fructose-6-phosphate phosphoketolase that is present in all bifidobacterial species. The ability of enzymes to utilize various saccharides in a biochemical test allows the clear identification of an isolate as a *Bifidobacterium* species based on a simple carbohydrate fermentation pattern (Mitsuoka 1969; Scardovi *et al.* 1969). Moreover, chemotaxonomic methods analysed the protein structure of specific cellular compounds. Whole-cell proteins profiling by Sodiumdodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for differentiating *B. adolescentis*, *B. bifidum*, *B. breve*, *B. dentium* and *B. longum* which were isolated from adult faeces, as well as for the identification of *B. lactis* from European probiotic products (Vitali *et al.* 2007). Proteomics identification is dependent on the protein characteristics and fingerprinting maps of cytoplasmic peptides that are generated by matrix-assisted laser desorption ionization-time of flight mass (MALDI TOF). Genotypic identification is based on information obtained from the genome or from specific genes rather than the expression products as in the case of phenotypic identification (Angelakis *et al.* 2011; He *et al.* 2007).

The rRNA sequence analysis is particularly used to assign relatedness of homology with the most related species among the genera. Molecular phylogeny is derived from a high correlation of elongation factors that are involved in the translation of diverse molecules of 16S RNA genes coding for 16S rRNA or 16S rDNA (Woese 1987). Several genotypic methods are based on the rule of polymerase chain reaction (PCR) which makes it possible for the specific amplification of targeted DNA fragments by using oligonucleotide primers. The generated oligonucleotide species-specific primer targets

sequences of the 16S rRNA gene for detection and identification of bifidobacteria to the genus and species or strain level (Kaufmann *et al.* 1997). The DNA sequence analysis is better than any other taxonomic method and is based on the amplification and cloning of 16S rRNA gene of DNA or RNA (cDNA). Selected clones from the microbial community are then sequenced and analysed in *silico* 'computer simulation'. Phylogenetic accession of bioinformatics tools are then applied which align and affiliate the clones to the closest relatives. Finally, the aligned sequences are grouped into a structure named molecular species or visualized in a phylogenetic tree. The molecular species either belong to a cultivated bacterial species or a bacterial species that has not yet been cultivated (Charalampopoulos *et al.* 2009; Stackebrandt *et al.* 1994).

## 1.1 Obesity

Obesity is one of the greatest public health problems and is caused by an excessive accumulation of fat to the point that it poses a risk to the individual's health (WHO 2011). The prevalence of obesity has noticeably increased in the past few decades and is responsible for more healthcare costs in comparison to any other medical condition. Obesity and overweight are mostly defined on the basis of body mass index (BMI). Overweight can be defined with a BMI of 25 $kg/m^2$ or higher while a BMI greater than or equal to 30 $kg/m^2$ is referred to as obesity. According to the WHO (2005), BMI is calculated as weight in kilograms divided by the square of height in metres, where the greater the BMI, the greater the hazard of comorbidities is; including diabetes mellitus, hypertension, obstructive sleep apnoea, several types of cancers, dyslipidemia, cardiovascular disease, and overall mortality. Obesity is a public health problem that has triggered worldwide concern. According to the World Health Organization (WHO), there are 2.3 billion obese people aged 15 years and above across the world; furthermore, over 700 million are expected to be obese by 2015 (Chan *et al.* 2010).

Overall, the policies for production of healthier food environments are recommended as a high priority for obesity prevention across the globe, but until recently, little action has been taken on this issue (Swinburn *et al.* 2013). The prevalence of overweight and obesity has been observed in the past few decades in many countries. It revealed that more than half of the American population are overweight and more than 30% are obese (Huttunen *et al.* 2013; Ogden *et al.* 2007). According to the German Health Interview and Examination Survey for Adults (Studie zur Gesundheit Erwachsener in

Deutschland), the percentage of overweight adults in Germany has not increased during the last decade but remained at a constantly high level, while the prevalence of obesity showed an upward trend, especially among young adults. Present information on the prevalence of overweight and obesity among German adults is not only based on the BMI index alone but also on the analyses of waist and hip circumference measurements (Mensink *et al.* 2013). An imbalance between energy intake and energy expenditure causes obesity which is not yet completely understood since it involves both genetic and environmental factors (Di Gioia *et al.* 2014). The reasons for going beyond obesity seem to be varied and involve genetic background, disequilibrium of energy homeostasis, a sedentary lifestyle, and other environmental factors (Friedman 2009). Nowadays, some authors consider obesity to be a transmissible disease because maternal obesity predisposes children to obesity in adulthood (Lawlor *et al.* 2006). The prevalence of obesity can become a worldwide epidemic among children and adolescents. In some studies, the association of obesity with a specific profile of the bacterial gut microbiota contributed to individual bacterial species and whole microbial communities (Tennyson *et al.* 2008).

## 1.2 Gut microbiota

Several factors cause obesity such as alterations in the body's regulation of energy intake, expenditure, and storage. Evidence from studies on animal models suggests that the gut microbiota influences nutrient consumption and energy regulation (DiBaise *et al.* 2008). The observations of studies on obese mice have been correlated with higher levels of Firmicutes and lower levels of Bacteroidetes phylum in comparison with lean examples. The mutated *ob/ob* mice were characterized by a 50% lowering of the amount of Bacteroidetes and a relative increase in Firmicutes for more than 5,000 bacterial 16S RNA gene sequences analysis from the gut microbiota compared to lean mice. This condition increases fermentation, facilitating more energy extraction in the host. Furthermore, feeding a high-fat diet to the mice showed a change in the microbiota structure with a decrease in the amount of predominant Gram-positive groups such as *Bifidobacterium* species (Ley *et al.* 2006). Moreover, the gut microbiota of lean individuals is more diverse than that of obese individuals (Turnbaugh *et al.* 2009).

The increase in energy consumption of less than 1% in comparison to the daily energy expenditure can cause an increase in body weight and metabolic syndrome in the long

term ‘through several years’ (Hill 2006). Gut microbiota raises the inhibition of fasting-induced adipose factor (FIAF). FIAF is one of the angiopoietin-like proteins expressed in the differentiated gut epithelial cells, liver and adipose tissue. The consequence of FIAF inhibition is the increased expression of lipoprotein lipase (LPL) activity that leads to a higher cellular gain of fatty acids and accumulation of greater fat storage in adipose tissue (Bäckhed *et al.* 2004; Flint *et al.* 2008). As a result, germ-free mice are resistant to diet-induced obesity. Germ-free mice were protected from gaining more weight and fat mass in comparison to the conventional mice when both were fed a (western diet) high-fat/high-carbohydrate diet. Surprisingly, another study revealed that germ-free mice as well as conventional mice had the same energy content in their faeces when both groups were fed a high-fat diet (Cani *et al.* 2009). The study result has not suggested more energy harvest from the high-fat diet in the conventional mice in comparison to the germ-free mice. The author proposed a mechanism according to which the gut microbiota could have participated in the regulation of AMP-activated protein kinase (AMPK) activity and the oxidation of fatty acids. AMPK activity is higher in the muscle even in the absence of gut microbiota, produces higher phosphorylation of its specific target acetyl-CoA carboxylase (ACC) and reduces the production of malonyl-CoA. The reduction of malonyl-CoA raises carnitine palmitoyl transferase-1 (CPT-1) which stimulates mitochondrial fatty acid oxidation, and therefore, the experiment result may be considered to be a bacterial-related mechanism more than energy harvesting that results in diet-induced obesity (Bäckhed *et al.* 2007).

A clinical trial has found that an increase in the accumulation of fat is the result of not only a positive energy balance and decreased physical activity, but also because of specific nutrition components linked to the host’s inflammatory state and the upward shift of the aggressive gut microbiota (Huang *et al.* 2013). Positive correlations have been found between energy intakes and plasma lipopolysaccharids (LPS) levels. LPS is a compound derived from the cell walls of Gram-negative bacteria which are members of the families Enterobacteriaceae and Desulfovibrionaceae in the phylum Proteobacteria. A high level of LPS is defined as metabolic endotoxemia which is related to gut, hepatic, and adipose tissue inflammation and the symptoms are more apparent with a high-fat diet rather than a normal chow diet in mice. An endotoxin activity of the previous pathogen families is a thousand-fold more compared to LPS from the family

Bacteroideaceae in the phylum Bacteroidetes (Chen *et al.* 2014; Zhao 2013). Alteration of the composition of the gut microbiota may contribute to the prevention as well as to the therapy of obesity (Vael *et al.* 2011). The analyses of intestinal microbiota composition have revealed that the plasma LPS concentrations correlate negatively with the dominant Gram-positive groups *Bifidobacterium spp*. and *Eubacterium rectale*, and *Clostridium coccidioides* groups. Moreover, the administration of *Bifidobactrium spp.* presented the ability to significantly reduce the content of intestinal endotoxin of the mice given high-fat meals (Cani *et al.* 2007). The altered gut microbiota in obese cases could have contributed to low-grade inflammation. A high-fat diet fed to mice causes metabolic endotoxemia, low-grade inflammation and increases macrophage infiltration in adipose tissue. The circulation of inflammatory markers are elevated such as IL-6, TNF-a, IL-8, IL-10 and these inflammatory factors are known to be involved in insulin resistance and excessive hepatic and adipose tissue lipid storage (Cani *et al.* 2007; Ley *et al.* 2006).

Bifidobacteria are present in the healthy neonate intestine after two to five days of birth and their levels rise within one week up to 99% of the amount of faecal flora (Mitsuoka 1984). Studies of bifidobacteria on human health have revealed their capability to adhere to the intestinal mucosa resulting in an inhibitory effect against a variety of enterotoxigenic, enteropathogenic and diarrheagenic bacteria (Bernet *et al.* 1993; Gleinser *et al.* 2012). *Bifidobacteria spp.* are considered to be probiotic and could stimulate the immune system and increase bioavailability of essential minerals that assist in digestion and nutrient absorption (Kumar *et al.* 2012). Probiotics are organisms that do not necessarily have a phylogenetic relation to one another, and are, therefore, best defined functionally rather than taxonomically (Rossi *et al.* 2011). Probiotics are defined as 'live microorganisms, which when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO 2001). Probiotic is also known as a prepared product including specific microorganisms in viably adequate numbers that are able to exert an influence on the microflora in the host with improved health effects (de Vrese *et al.* 2008).

In Japan, the first applied research on Bifidobacteria as a probiotic started in the 1950s. The Morinaga Milk Industry Company advanced 'bifidus®'-fermented milk products containing *Bifidobacterium longum* and *Streptococcus thermophilus*. Later, technologies

were launched so that the market products by 1978 contained Bifidobacteria such as Mil-Mil®-fermented milk products that contained *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Lactobacillus acidophilus* which had been proved to lead to an improvement in the health of the general public by Yakult® in Japan (Ishibashi *et al.* 1993). The probiotic prepared products that contain bifidobacteria are varied depending on the different dairy products. These days, commercial probiotic products contain a single genus *Bifidobacterium* or in combination with other lactic acid bacteria such as lactobacilli and *Streptococcus thermophiles.* The genus Bifidobacterium have encompasses potential probiotics: *B. adolescentis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve* and *B. longum* subsp. *infantis* and *B. longum* subsp. *longum.* The probiotic products containing bifidobacteria contribute to a large share of the market value, with approximately $1.3 billion in Europe alone (Leatherhead Food Research Association 2001).

# 2. Material and Methods

## 2.1. Subjects and sampling

The stools from sixteen healthy breastfed, infants were collected in sterile vials. None of the infants suffered from gastrointestinal or any major illness or received antibiotic therapy. The sampling was approved by the children's hospital (Kinderklinik Giessen), and their parents' agreements were obtained. All samples were delivered to the laboratory which occurred within half an hour after collection.

## 2.2. Pre-reduced peptone water preparation

Pre-reduced peptone water (g/100ml) was prepared by weighing (Mettler AE163, Germany) peptone 0.1 g (Merck, Darmstadt, Germany), NaCl 0.85 g (CarlRoth, Germany), L-Cycteine-HCl 0.05 (Sigma, Japan) and dissolved it in deionized distilled water. The contents were mixed well using a magnetic stirrer at 300 rpm/5 min (Heidolph MR3001, Germany) and adjusted to pH: 7. After separating pre-reduced peptone water in test tubes and containers, the proper diluents were sterilized by autoclaving (Webeco-Wolf, Germany) at 121°C for 15 min (Tzortzis et al. 2005).

**List of instruments**

| | |
|---|---|
| Anaerobic Gas Jar | BBL-Difico, USA |
| Autoclave | Webeco-Wolf, Germany |
| Balance | Mettler AE163, Germany |
| Centrifuged | Biofuge primor, Heraeus-Germany |
| Contrast Phase Microscope | Axiophot 2–ZEIZZ, Oberkochen–Germany |
| Freeze-dryer | Lyova GT2, Steris-Germany |
| Magnetic Stirrer | Heidolph MR3001, Germany |
| pH-meter | Heidolph, Germany |
| Spectrophotometer | Thermo-Spectronic-Genesys 10UV, Fisher, USA |
| Steam Boiler | Medizin & Lab. Tech. Fritz Goessner, Hamburg-Germany |
| Thermal Cycler PCR | BioRad, Germany |
| UV-light Bands visualizer | BioRad Gel Doc2000-Germany |
| Vortex | Heidolph-real, Germany |

**List of materials**

| | |
|---|---|
| ALT kit | Alanine aminotransferase |
| $CaCl_2.6H_2O$ | Oxoid Ltd. Basingstoke, UK |
| $CO_2$ gas generator kit | Anaerocult A, Merck-Darmstadt, Germany |
| Control diet (C1090-10) | Altromin-Spezialfutter, Germany |
| DNA marker | Fermentas-Thermo Scientific, Germany |
| DNeasy blood and tissue kit | Qiagen, Hilden, Germany |
| EDTA | Merck- Darmstadt, Germany |
| Glucose | Merck, Darmstadt-Germany |
| High fat diet (C1090-70) | Altromin-Spezialfutter, Germany |
| $K_2HPO_4$ | Oxoid Ltd. Basingstoke, UK |
| $KH_2PO_4$ | Oxoid Ltd. Basingstoke, UK |
| L-Cysteine-HCl | Sigma, Japan |
| Male C57BL/6JRj mice | Janvier, 53941-ST-Berthevin, France |
| $MgSO_4.7H_2O$ | Oxoid Ltd. Basingstoke, UK |
| Micro-filter | FP30/0.2CA-S Whatman, Germany |
| MRA agar | Merck- Darmstadt, Germany |
| Mupirocin | Sigma, USA |
| NaCl | CarlRoth, Germany |
| Oligofructose | Merck, Darmstadt-Germany |
| Oligofructose | Spennrad, Germany |
| Peptone | Merck, Darmstadt, Germany |
| Reflotron Analyser | Roche Basel, Switzerland |
| Skim milk | Merck, Darmstadt-Germany |

## 2.3. Media composition

Empty screw cup bottles (100ml) were autoclaved (Sanoclav-Wolf, Germany) at 121°C for 20 min and filled with 30, 40 and 80 ml deionized water in Erlenmeyer flask. Weighed 30, 40 and 80 mg mupirocin antibiotic (Sigma, USA) and transferred into Erlenmeyer flasks contained 30, 40 and 80 ml. The solutions were sterilized through a 0.2 µm filter (FP30/0.2CA-S Whatman, Germany). The antibiotic stock solutions for three different concentration mupirocin MRS agar media were stored in the fridge (Ferraris *et al.* 2010). Three selective 30, 40 and 80 mg/l mupirocin MRS media for *Bifidobacteria* were prepared by dissolving 74.5 g of MRS (Merck- Darmstadt, Germany) media and 0.5 g L-Cysteine-hydrochloride in one Liter of deionized water. Complex agar media composition of MRS was (Casein peptone 10 g, Yeast extract 5 g, D-glucose 20 g, $KH_2PO_4$ (6 g), $NH_4$-Citrate 2 g, Tween 80 (1 ml), Na-Acetate 15 g, $MgSO_4$ (0.575 g), $Fe^{+3}$-

sulfate 0.034 g, $MnSO_4$ (0.12 g), Agar-Agar 15 g. The bottles were steamed for one hour at 100°C as recommended by manufacturer. The medium was cooled to approximately 65°C and 1.3 ml per liter of acetic acid 96% was added, while the pH value was approximately 5.5. Three different concentrations of mupirocin were added and the final mupirocin MRS media were poured into the plates (Thitaram *et al.* 2005; Ferraris *et al.* 2010).

A sample of one gram of feces was dissolved in sterile 100 ml pre-reduced peptone water and vortexed for 5 min (Heidolph-real, Germany). Serial dilutions of samples were prepared and the $10^{-5}$ – $10^{-7}$ dilutions was spread on MRS agar plates in triplicate. All plates were incubated anaerobically using anaerobic gas jars (BBL-Difico, USA containing gas packs), and added 35 ml of distilled water was added for the production of an anaerobic environment (Anaerocult A, Merck-Darmstadt, Germany). Oxygen binds quickly and completely, creating an oxygen-free environment and a $CO_2$ atmosphere generates in a 2.5 liter anaerobic jar. Plates were incubated for 3 days at 37°C. After incubation on the mupirocin (80 mg/L) MRS media, white colonies were counted.

The morphology of bifidobactria growth was examined by contrast phase microscopy (Axiophot 2–ZEIZZ, Oberkochen–Germany). After incubation white colonies were counted from (80 mg/l) mupirocin MRS media. Several typical colonies (2-5 mm diameter) were picked randomly from plates and sub-cultured on MRS medium and incubated anearobically at 37°C for three days.

## 2.4. Identification of Bifidobacteria

Single colonies (2-5 mm diameter) formed on the 80 mg/L mupirocin MRS media were picked with a sterilized toothpick and suspended in 1µl PCR special water. The identification of typical morphological shapes of bifidobacteria based on phenotypic traits was performed using contrast phase microscopy technique (Matsuki et al., 2003). Morphological observations were smeared for identification typical bifidobacteria shape based on phenotypic traits by contrast phase microscopy technique (Matsuki et al., 2003). The suspended colony mixed with 180 µl TE buffer (10 mmol/L of Tris–HCl, 1 mmol/L of EDTA; pH 8) containing lysozyme (1mg/mL) and incubated for 1 h at 37°C. Finally, 10 µl proteinase K was added to the suspension and incubated for 2 h at 56 °C. DNA was subsequently isolated using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The PCR reaction mixture consisted of 2.5 µl buffer (10mM Tris-HCl (pH7.6) and 1mM EDTA), 2.5 µl mM dNTP's, 0.5 µl MR Primer MrF/MrR were primer 1(10 p mol) and 0.5 µl primer 2(10 p mol), 17.9 µl water and Tag- Polym + 1 µl DNA. The marker used (Lambda DNA/ EcoRI+ Hind III Marker, Fermentas-Thermo Scientific, Germany) on the site 1500 bp (16.3ng/0.5 µl) 3.3%. Water and *E. coli* were negative and positive control respectively. The thermal cycling (BioRad, Germany) conditions were an initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 sec, annealing at 57.3 °C for 45 sec, extension at 72 °C for 2 min, repeated for 29 cycles, and final extension at 72 ° C for 4 min. For the detection bands used agarose gel, 0.5 µg/Lane, 8 cm length gel, 1xTAE, 7v/ cm and 45 min). Agarose gel prepared 1% (0.8g / 80ml $H_2O$) and the solution heated in microwave with high power energy and poured into the device chamber with wells temple maker (comb). After solidified gel sampled in each well mixed the dye with glycerol to residue the dye in the button of wells. Bands visualized with ethidium bromide (0.5 µg/mL) under UV light (wavelength 260 nm, BioRad Gel Doc2000-Germany) (Vlokova *et al.* 2004).

These two isolates were identified as *B. breve* M4A and *B. longum* subsp*. longum* FA1 by the amplification and sequencing of the 16S rRNA gene using the oligonucleotide primers EUB9F (5`-GAGTTTGATCMTGGCTCAG-3') and EUB1492R (5`-ACGGYTACCTTGTTACGACTT-3') (Lane 1991). The PCR reactions were performed as described by Kampmann *et al.* (2012) and the amplicons were sequenced by the company LGC Genomics (Berlin, Germany). Next relatives' sequences were obtained by homology searches using BLAST algorithm available at http://blast.ncbi.nlm.nih.gov/Blast.cgi and the NCBI database. The accession number was CP006716.1 and KM577186.1 for *B. breve* M4A *and B. longum* subsp. *longum* FA1, respectively.

*Bifidoacteria* subspecies were identified by growth on basal medium. The medium consisted of (g/l): peptone water (Oxoid Ltd. Basingstoke, UK) 2, yeast extract (Oxoid) 2; NaCl, 0.1; $K_2HPO_4$, 0.04; $KH_2PO$, 0.04; $MgSO_4.7H_2O$, 0.01; $CaCl_2.6H_2O$, 0.01; $NaHCO_3$ 2,; L-cysteine HCl (Sigma) 0.5, and (1% w/v) media were tested with glucose, L-arabinose and ribose (filter sterilized and added after autoclaving). The component media were dissolved in and then were flashed $CO_2/N_2$ gas into 10 ml cupped bottles for 5 min. The bottle media were further autoclaved at 121°C for 20 minutes, once cooled

the carbohydrates were filtered and added to the media to make an overall concentration of 1% (w/v). From MRS broth medium grown, one ml of *B. longum* subsp. *longum* FA1 was injected aseptically into each tube and incubated at 37°C. Each species was grown on each carbohydrate in triplicate. A blank containing medium with no added carbohydrate was also inoculated for each bifidobacteria species in triplicate, in order to take into account any carryover of glucose. The growth was measured (Thermo-Spectronic-Genesys 10UV, Fisher, USA) at a wavelength of (650 nm) at 0, for 72 hours post inoculation (Palframan *et al.* 2003; Sakata *et al.* 2002).

## 2.5. Samples preparation

Special attention was paid to the beginning of the early stationary phase. The optical densities were measured of *B. longum* subps*. longum* FA1 and *B. breve* M4A cultures versus time to obtain maximum growth curves. The Bifidobacterium strains incubated until the stationary phase were reached in MRS broth media. In the cup 100 ml bottles 5% starter culture was flashed with $N_2/CO_2$ gas for 5 min and incubated at 37 ºC for 72 hr. The growth of the cells was followed by measuring the pH (pH-meter Heidolph MR3001, Germany) and optical density of the culture at 580 nm using a Spectrophotometer (Thermo Spectronic Genesys 10UV, Fisher -USA) in three different times (Trsic-Milanovic *et al.* 2001).

The culture strains of 100 ml MRS broth media in their stationary phase were centrifuged (Biofuge primor, Heraeus-Germany) at 2500 × *g* for 10 min at 4 ºC. The supernatant was discarded and the concentrated cells were suspended in 20 ml of skim milk (10%) and glucose (5%) media (Merck, Darmstadt-Germany).The medium was steamed at 100 ºC for 30 min (Medizin & Lab. Tech. Fritz Gossner, Hamburg-Germany). The suspension was separated into 1 ml for each vial. The first phase was lyophilization by freezing the vials at -18 ºC overnight and the second phase was lyophilizion (Lyova GT2, Steris-Germany) by freezing at -18 ºC, vacuum -2.7 mbar for 14 hour. After freeze drying, the vials were sealed under vacuum and stored in a refrigerator (Trsic-Milanovic *et al.* 2001; Carvalho *et al.* 2004).

The viable cells (CFU/mL) of bifidobacterium strains were enumerated by the MRS agar plate count method after dilution in pre-reduced peptone water. The plates were incubated under anaerobic conditions at 37 ºC. The colonies were enumerated and recorded as colony forming units per milliliter from lyophilized strains by added 1 ml

sterilized distilled water into each lyophilized vial for 30 min. The cultures obtained after suspension of lyophilized bacterial cells were diluted in pre-reduced peptone water. The survival rates of the cultures after lyophilization were estimated in terms of the number of surviving cell counts. The results were arithmetic means of three measurements.

After 6 months of storage growth activity of freeze-dried strains were measured by enumerated on MRS agar plates. Freeze-dried preparations were rehydrated with 1 mL of sterile water into each lyophilized vial for 30 min, diluted and plated. After incubation of the plates under anaerobic conditions as described above the survival rates of the cultures after lyophilization were estimated in terms of the number of surviving cell counts recorded as colony forming units per mL (CFU/mL). The results were arithmetic means of three measurements.

## 2.6. Culture media

For growth improvement and better survival of *B. longum* subsp. *longum* FA1 and *B. breve* M4A the strains were cultured in 10% reconstituted skim milk media which was supplemented with different concentrations of two carbohydrates and yeast extract. For this, skim milk (10%) powder was dissolved in distilled water and supplemented with 0.3% or 0.6% yeast extract and oligofructose or glucose at different levels (1%, 2% and 3%). Skim powder (10%) without addition was used as control. The skim milk media was heat treated at 110˚C for 10 min. Skim milk media were inoculated with a 10% (v/v) *B. longum* subsp. *longum* FA1 and *B. breve* M4A culture after activation of lyophilized culture and were incubated anaerobically for 48 h at 37°C. Skim milk media samples were withdrawn for pH and turbidity measurements. For the enumeration of viable cells, skim milk media were serially diluted in 0.1% (w/v) sterile pre-reduced peptone water and plated in triplicate onto MRS agar. Plates were incubated anaerobically at 37°C for 3 days. Viability was expressed as log CFU/mL.

An aliquot of skim milk was taken at intervals (1, 5, 25, 35, 45 hours) and diluted (1:10 v/v) with 0.2% (w/v) EDTA, (pH 12.0) and turbidity was measured (Thermo-Spectronic-Genesys 10UV, Fisher, USA) at 640 nm. Un-inoculated milk media were diluted with 0.2% (w/v) EDTA and used as a blank. Specific growth rate (μ) for each culture was calculated using the following equation: $\mu = (\ln X_2 - \ln X_1) / (t_1 - t_2)$, where $X_2$ and $X_1$ corresponds to the cell density at time $t_2$ and $t_1$. Doubling time (Td) was calculated as: $Td = \ln 2 / \mu$ (Shin *et al.* 2000).

In order to obtain the desired number of bifidobacterium strains, the medium was supplemented with carbohydrates to enhance its growth and survival. Skimmed milk powder containing 10% solids (Merck) was reconstituted in distilled water. Skimmed milk base (containing 0.3% yeast extract) was supplemented with 3% (w/v) oligofructose for culture of *B. longum* subsp*. longum* FA1 and 3% glucose for *B. breve* M4A. The media were separated in capped bottles containing 10 mL media, flashed with $CO_2/N_2$ gas into bottles and sterilised by autoclaving at 121°C for 10 min, and inoculated with 10% (v/v) *B. longum* subsp*. longum* FA1 or *B. breve* M4A cultures after the activation of each pure lyophilised culture for 24 h. The cultures were incubated anaerobically at 37°C, and the pH was adjusted: ~5 mL samples were withdrawn every 16 h for analysis.

## 2.7. Design of the animal experiment

An obesity-inducing high-fat diet leads to hepatic steatosis (Yaqoob *et al.* 1995). The induction of obesity is most effective when started at a young age and continued for several weeks (Peckham *et al.* 1962).

During the first week of the study, mice in the high fat diet-*B. longum* subsp. *longum* FA1 (HFD-FA1) and high-fat diet *B. breve* M4A (HFD-M4A) groups were given a simple control diet for adaptation. From week 2 to 6 both groups were supplemented with *B. breve* M4A and *B. longum* subsp. *longum* FA1 with oligofructose administered by gavage orally. A combination of prebiotics and probiotics was used in order to increase the survival of the bifidobacteria in the intestine (Manning *et al.* 2004). A third group received a high-fat diet only (HFD).

The interaction between body composition and nutrition differs among mouse species. In addition, different diets elicit different reactions towards obesity among mice (Jürgens *et al.* 2006). The effects expected in the current study were estimated according to (Kondo *et al.* 2010), who performed a study for >8 weeks but detected effects after 6 weeks. The minimum number of mice was calculated (1) based on a significant difference in epididymal fat pad between the control and B-3H groups. (2) Significant differences in weight gain between the control and *Bifidobacteria breve* 3H groups were identified after 6 weeks.

The alpha error was set at 0.05 (two-sided) and the tolerated beta error was 0.2. No correction for multiple comparisons was performed, and calculations were based on parametric tests (one-way ANOVA with LSD post hoc test). The calculations were

performed using '3.1 GPower©' (University of Duesseldorf, Germany). Six animals per group were needed for the minimum sample size based on the above assumptions. In addition, variance heterogeneity might result in higher scatter of the measured values. Therefore, we increased the number of mice per group to seven.

## 2.8. Establishment of animal experiment

The district president of Giessen approved the study protocol. All experiments were performed under specific pathogen-free conditions. Mice were purchased and housed in specific barrier facilities according to the Federation of Laboratory Animal Science Associations (FELASA) recommendations. Each cage was closed using a filter top and was ventilated individually with modified barrier-systems (individually ventilated cages-IVC). Cages were only opened under laminar flow conditions at the cage changing station.

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

The drinking water of mice in the intervention groups (HFD-FA1 and HFD-M4A groups) was supplemented with 0.1 g/day oligofructose (Spennrad, Germany) based on a water consumption of 4 mL/day per mouse (25g/L oligofructose in water). The purpose of oligofructose supplementation was to aid the administered bifidobacteria to survive and adhere to mice intestinal mucus. The capability of bifidobacteria strains to adhere to the host intestinal mucosa is necessary for therapeutic manipulation and the adhesion has the capacity to be a strain dependent (Collado *et al.* 2005). Bifidobacteria administration was started by the second week of the experiment. The administration was given in the light phase.

A single dose of 0.20 mL/day was administered orally by gavage to each mouse. For the growth improvement and better survival of *B. longum* subsp. *longum* FA1 and *B. breve*

M4A, the strains were cultured in 10% reconstituted skim milk media. The media were supplemented with different concentrations of two carbohydrates and yeast extract. Skimmed milk was supplemented with 0.3% yeast extract and 3% oligofructose for *B. longum* subsp*. longum* FA1 or 3% glucose for *B. breve* M4A.

Table 1: Composition of experimental diets

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

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

The fermented milk cultures administered to the HFD-FA1 and HFD-M4A groups contained $2.9\times10^6$ CFU/day of *B. longum* subsp*. longum* FA1 and $4.1\times10^6$ CFU/day of *B. breve* M4A, respectively. For establishing an equal stress, the HFD group was orally (by gavage) given the same dose of skimmed milk supplemented with 0.3% yeast extract and 3% glucose as the intervention groups. The food intake and the mice were both weighed weekly using a balance (Ohaus-Explorer Pro, Switzerland). The different groups of animals were fed as follows:

1. The HFD group was fed a high-fat diet.
2. The HFD-FA1 group was fed a high-fat diet and received *B. longum* subsp*. longum* FA1.
3. The HFD-M4A group was fed a high-fat diet and received *B. breve* M4A.

At the end of the study, mice were euthanized under $CO_2$ with humane endpoints.

Food intake (FI), 'the amount of food ingested by the animals in each group' was calculated. Mice were moved to clean cages and the amount of food given to them was

weighed. Any food remaining in the previous cage was also weighed. These measurements were repeated weekly, and the FI (g/day per mouse) was calculated using the following equation according to (Feige *et al.* 2008):

$$FI\ (g/day\ per\ mouse) = (W_0 - W_{ed}) / (n_d \times n_m),$$

$W_0$ = weight (g) of the food provided,

$W_{ed}$ = weight (g) of the food remaining in the cage at the end of the feeding period,

$n_d$ = number of days over which FI was calculated,

and $n_m$ = number of mice per cage.

Serum samples were stored at −80°C until used. The total serum cholesterol, triglyceride and alanine aminotransferase (ALT) levels were measured using a Reflotron analyser (Roche Basel, Switzerland) following the manufacturer's instructions. The Reflotron analyser was calibrated using calibrating strip and then 32-µL serum samples were added to strips for measurement of ALT, triglycerides and cholesterol. The Reflotron instrument automatically detected the measurement type using magnetic tape fixed on the strips.

## 2.9. Pathology

### Lipid extraction and sample preparation

Approximately 50 mg of liver tissue was weighed and transferred into a 2 ml Eppendorf cup tube (Chem solute & VWR, Germany) that contained 500 µl of n-hexane: isopropanol (3:2, v/v; (Hara *et al.* 1978) .The liver tissue was disrupted with a bead mill (Tissuelyser II - Qiagen, Hilden, Germany) for 3 min at 20 Hz in a mixer mill. The tissues were homogenized by shaking (Eppendorf mixer, type 5432, Wesseling, Germany) for 30 min. The homogenate sample tubes were centrifuged at 1000×g (Concentrator plus-Eppendorf, Germany) for 10 min at 4°C. The supernatant was transferred to a new tube, and the bottom residue was discarded. The supernatant can be stored in a tight cup tube at -20 °C (Rodríguez-Sureda *et al.* 2005). Aliquots of the lipid extracts were dried and the lipids solubilized using a 1:1-mixture of chloroform and Triton X-100 (De Hoff *et al.* 1978).

### Triglyceride and cholesterol measurement

Triglycerides and cholesterol of the liver were determined with colorimetric quantification Fluitest® kits (Analyticon- Biotechnologies AG, Germany) measured the absorbency at

546 nm (Cary 50BIO-Varian, Darmstadt, Germany). The measurements were performed with test kits TG: triglycerin (method: GPO-PAP, No. 5741) and cholesterol (method: CHOD-PAP, No. 4241) for triglyceride and cholesterol, respectively.

## Bacterial count of the large intestine content

The cecum and a portion of the adjacent colon tissue of each mouse were removed and placed in capped sterile tubes. After transfer to a laminar flow cabinet, 1 gram of each sample contents was transferred to a tube with 9 mL of Ringer (Sigma-Aldrich) solution (1/4 power) and homogenized by vortexing for 1 minute. Eight to nine fold serial dilutions of each sample were performed which were plated with selective mupirocin 100 mg/L MRS agar medium for bifidobacteria count and the MRS agar medium was used for lactic acid bacteria count. Both media were incubated anaerobically at 37°C for 72h in triplicate. The numbers of CFU were expressed as log CFU/g.

## Histology of liver tissues

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

## Haematoxylin and eosin staining

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

## Fixation and dehydration

Fixation in para-formaldehyde is used to protect the tissues against bacteria and enzymatic degradation. The conserved structure of the tissue was used for fatty liver examination.

## Phosphate buffered saline PBS (1x) preparation

PBS buffer saline solution were prepared from 8.0g NaCl, 0.2g KCl, 1.42g $Na_2HPO_4$ (Or $Na_2HPO_4$. $2H_2O$), 0.27g $KH_2PO_4$, and dissolved in 950 ml distilled water at pH value 7.4. The volume was completed to 1 liter and the pH checked again.

## Paraformaldehyde preparation

Almost 1g of PFA was dissolved in 100ml PBS (1x) under warming conditions with 1N NaOH (about 5 drops). It was important the pH value doesn't increase above 8. After liver samples were weighted and taken out, the liver tissues were cut off 5mm×5mm×5mm piece. Probes were fixed overnight in fixation cassette in 1% PFA/PBS. Next day samples were washed extensively and embed in paraffin.

## Dehydration of tissue

Liver tissue was placed in different solutions of the Enclosed Tissue Processor (Leica ASP 300S, Germany) of ethanol, xylene and paraffin (table 2). The tissues remained in xylene until they became transparent. Liver tissue was transferred into plastic embedding cassettes. Tissue samples were placed in a steel tray, partly bottom filled with liquid paraffin then immersed in a paraffin bath (Leica RM 2165, Germany). The cassette was placed on top of the steel tray and was completely filled with liquid paraffin.

Table 2 : Dehydration of the liver tissues

| **Step** | **Solution** | **Time** |
|---|---|---|
| 1 | 70% ethanol | 30 minutes |
| 2 | 96% ethanol | 1-2 hours |
| 3 | 96% ethanol | over night |
| 4 | 100% ethanol | 30 minutes |
| 5 | 100% ethanol | until end of day |
| 6 | 100% ethanol | over night |
| 7 | 100% ethanol + a drop of eosin | 1 hour |
| 8 | 50/50 xylene and 100% ethanol | 30 minutes |
| 9 | xylene | 1-5 minutes |
| 10 | liquid paraffin | 3 hours |
| 11 | liquid paraffin | 3 hours |
| 12 | solid paraffin | over night |

The paraffin was solidified (Leica EG 1150 C, Germany) at -18°C for one hour. Sectioning was performed with a microtome (Leica RM 2165, Germany). Sections of the paraffin embedded tissue were cut off into 3 µm using a microtome blade (Feather A35, Japan) and transferred to a water bath at 40 °C.

The warm water made the sections stretch out before they were transferred to glass slides (Super-Frost Ultra plus, Germany). The slides were dried at 30°C (Memmert, Germany) and then placed in a box for subsequent staining (Lynch *et al.* 1969).

## Staining with hematoxylin and eosin

Slides of tissues (table 3) were stained in order to examine the cells under a microscope. The dyeing reaction of Hematoxylin and Eosin on the nuclei and cytoplasm, respectively, made it possible to differentiate cell types and determine fat and cell size. The tissues were rehydrated with water before staining. After staining with both colorants, the tissues were dehydrated with ethanol and xylene before they were sealed with mounting medium. The tissue slides were put into racks which were placed in a number of glass baths. These glass baths were filled with the liquids. After the staining process, the slides were mounted with the Pertex (Merck, Germany) and a thin glass slide was placed on top.

Table 3 : Dehydration of tissue before hematoxylin staining for paraffin sections

| **Incubation time (min)** | **Reagent** | **Note** |
|---|---|---|
| 40-60 | 60°C | in an incubator |
| 10 | xylene | |
| 10 | xylene | |
| 5 | xylene | |
| 5 | Ethanol abs. 99,6% | |
| 5 | Ethanol abs. 99,6% | |
| 5 | Ethanol abs. 96% | |
| 2 | Ethanol abs. 70% | |
| 5 | Tap water | |
| 2 | Hämalaun- solution | ready for use after Mayer |
| 5 | tap water | |
| 15" | Eosin-Lösung | ready for use of water soluble Eosin |
| < 1' | tap water | |
| 2 | Ethanol 96% | |
| 5 | Isopropanol | |
| 5 | Isopropanol | |
| 5 | xylene | |
| 5 | xylene | |
| 5 | xylene | |
| | Cover sliding with Pertex | |

## Oil Red O Staining

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

Oil Red O staining was used to detect lipid accumulation in liver tissues. To produce the stock solution, 0.5 g of Oil Red O was dissolved in 100 mL isopropanol with gentle heating in a water bath. Fresh stain was prepared for each experiment.

Ten-micrometre sections were cut and then air-dried. Slides were fixed in 10% formalin or 4% paraformaldehyde and washed briefly with running tap water for 1–10 min.

## Mayer's Hematoxylin Solution

The Mayer's Hematoxylin solution was purchased from Carl Roth-76185 Karlsruhe, Germany.

## Oil Red O Stain (0.5%) solution

Oil red O powder…….0.5 g

60% isopropanol……100.0 ml

A small amount of propylene glycol was added to the oil red O and mixed well. The remainder of the propylene glycol was gradually added and periodically stirring. The solution was gently heated until the solution it reaches 95°C. (The solution was avoided to go over 100°C.) Stir while heating. Filter through coarse filter paper while still warm. Allowed to stand overnight at room temperature. Filter again before use.

## 60% Isopropanol Solution

Isopropanol…….60.0 ml

Distilled water……40.0 ml

Note: The oil red O solution and 2 Coplin jars of 60% i*sopropanol* glycol were stored an oven at 60°. They were then rinsed with 60% isopropanol and stained with freshly prepared Oil Red O working solution for 15 min. After rinsing briefly with 60% isopropanol, the nuclei were stained lightly with alum haematoxylin. Additionally, the slides were rinsed with distilled water, and mounted in Dako® fluorescent mounting medium (Dako, USA) and covered with coverslips (Koopman et al., 2001; Speranza and Fail, 2005).

### Staining Procedure

- Dip briefly in distilled water.
- Dip slides in undiluted isopropanol.
- Stain in oil red O solution for 1 minute at 60°C.
- Differentiate by dipping twice in each of 2 changes of 60% isopropanol at 60°C.
- Rinse in 2 changes of distilled
- Counterstain in Mayer's hematoxylin for 30 seconds. Be sure to filter hematoxylin prior to use if not filtered daily.
- Rinse in distilled water. Do not use acid alcohol. Do not dehydrate through alcohol.
- Mount in an aqueous mounting medium.
- Results: Fat……Intense red, nuclei ……Blue

The staining of the lipid droplets were visualized using a light microscope (LEITZ DMRB-Leica, Germany) at 100X magnification. Pictures were taken with a digital camera (MXA 5400-Nikon) and setting it on 1.6 second, shutters F4.6, brightness light 8, visibility (50%:50%). Lipid droplets size in microscopy images were determined by Image J software program (2 x 2.1.7.4). The photos analysed with binary setting and the area calculated. Dirty or air bubbled photos were avoided.

## 2.10. Statistical analyses

All experiments were repeated three (two for doubling time) times. The analyses were performed in dublicate. Statistical analyses were performed using SPSS version 22.0.0.1 (IBM software, USA). Analyses of variance (ANOVA) was used for multiple comparisons for doubling time, and $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively. The post hoc least significant difference (LSD) test was chosen for homogenous variances. To assess the relationship between yeast extract, oligofructose, glucose and the doubling time we used three factorial Analyses of variance ANOVA making use of the mixed procedure in SPSS program to account for heterogeneity of variances in an unbalanced design.

The mediation analysis of indirect effects media supplemented with yeast extract, glucose and oligofructose affected on count number and acid production. The indirect influence of the skimmed milk media affected on pH value in such nonlinear model. The

relationship were evaluated between Medium (yeast extract, oligofructose and glucose) on pH via count number as a mediator using multiple, nonlinear regression models. Multiple Regression analyses of dependent factors of pH value and count number. The factorial design of our analysis was nonlinear relationship between independent and dependent variables.

$$(1)\ y = a + b_1 * x_1 + b_2 * x_1^2 + b_3 * x_2 + e$$

$$(2)\ y = a + b_1 * x_1 + b_2 * x_1^2 + e$$

With y = dependent variable (here: pH), a = constant, e = error term, $b_1$ ... $b_3$ = regression coefficients, $x_1$ ... $x_2$ = independent variables (glucose and oligofructose and count number), $x_1^2$ = $x_1$ squared (power term for nonlinear part of the model). The unbalanced present or not some ingredient and more or less concentration of glucose, and oligofructose with heterogeneity of variance, therefore, we cannot run p value and trust with it. Another ANOVA was run to its hidden and mix procedure accounting for the heterogeneity of ANOVA and correcting the *p* value.

All experiments were repeated three times independently with randomized designs. The analyses were performed in triplicate. Statistical analyses were performed using SPSS version 22.0.0.1 (IBM software, USA). Comparison of the bacterial number was made after logarithmic transformation. Pairwise of the single comparisons was used (each treated group to the HFD group), and $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively. The post hoc LSD test was chosen for homogenous variances; in cases with heterogeneity of variances, Tamhane's $T_2$ test is performed. For data sets with outliers, medians were analysed instead of means and the median test was used.

# 3. Results

## 3.1. Media selectivity

Typical morphological cell shapes of *Bifidobacterium spp.* were distinct from non-*Bifidobacterium spp.* Figure (2) shows bacteria from infant feces under contrast phase microscope in MRS agar media. The supplemented media with different mupirocin concentrations 30, 40, and 80 (mg/L) respectively, showed different levels of visual differentiation or selectivity of presumptive *Bifidobacterium spp*. from non-bifidobacteria. Figure (2A) showed the typical *B. infantis* shape growth in the mupirocen 30 (mg/L) from pure culture.

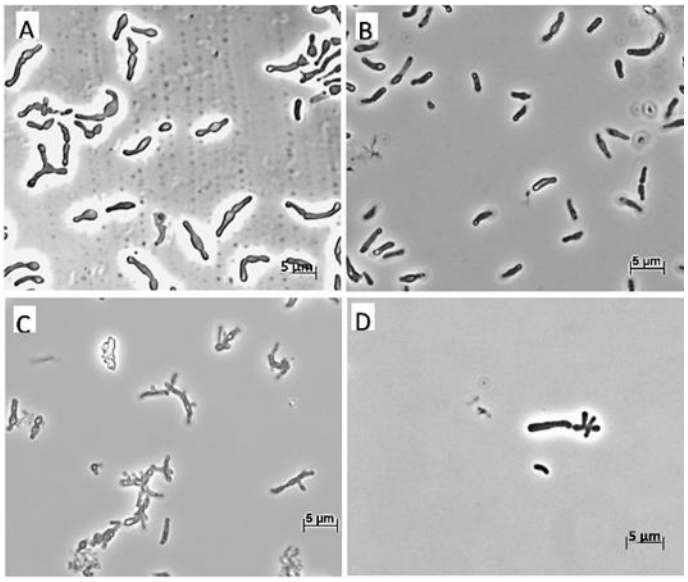


**Figure 2: Bifidobacteria and non-bifidobacteria from infant feces in contrast phase microscopy (100x) after growth on MRS agar media. (A): Typical cell shape of B. infantis (DSMZ no. 20088); (B): Low selectivity (30 mg/L mupirocin); (C): Selective (40 mg/L mupirocin) and (D): High selectivity (80 mg/L mupirocin).**

The mupirocin concentration 30 (mg/L) in figure (2B) showed inconsistent selectivity against non-bifidobacteria species. The level 40 mg/l of mupirocin figure (2C) was considered a visual differentiation of presumptive *Bifidobacterium spp*. from non-bifidobacteria which enhanced at this level of mupirocin. While figure (2D) supplemented with 80 (mg/L) mupirocin resulted in a higher selectivity and readily distinguishable presumptive bifidobacteria cell morphology.

## 4.2. Bacterial counts

Bacterial growths from infant feces were determined from colony-forming unit counts. Figure (3) showed white rounded colony. These colonies were presumptive for bifidobacteria in the highest level of mupirocin MRS agar media.

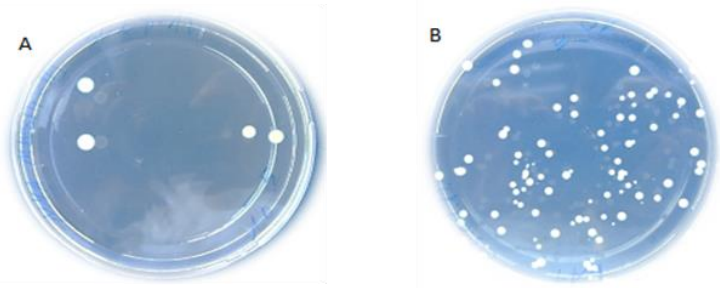


**Figure 3: Mupirocin (80 mg/L) MRS agar plates inoculated with infant feces, incubated anaerobically at 37 ˚C for 72 h. Colony morphology examples indicating typical Bifidobacterium spp.**

Cell numbers of CFUs have been determined from feces of infants. In figure (4) the CFU of a newborn baby (one day old) was non-detected.

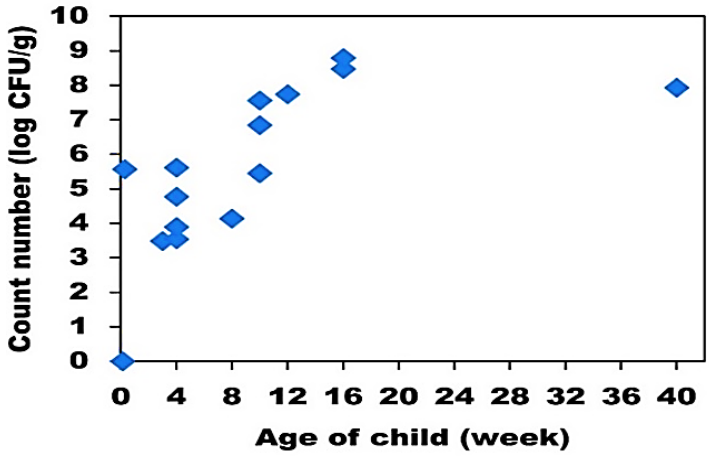


**Figure 4: White colonies forming units of Bifidobacteria growing on MRS agar media with mupirocin (80 mg/L) from feces of breast fed infants at different ages.**

From a two day old, a CFU of 5.57 log /g has been detected whereas after an age of three and four weeks 3.48, 3.54, 3.89 and 4.77 CFU log/g has been obtained, respectively.

The CFUs of 6.84 and 7.56 CFU log/g were determined in feces of 10 weeks old babies. The after four and ten months the CFU remained similar 8.47, 8.79 and 7.93 log CFU/g, respectively. It has been reported earlier that the number of bifidobacteria increased with age of the baby (Roger *et al.,* 2010; Serafini *et al.,* 2011).

## 3.2. Identification of Bifidobacteria isolates

Three selected bifidobacteria isolates have been identified at the genus and species level. First, the gene for 16S rRNA was amplified by PCR techniques using specific primers. Figure (5) showed the amplified nucleotide sequences which were run on a 1% agarose gel electrophoresis and the amplified DNAs appeared in the region 1500 bp. The strains showed high level of phylogenetic similarity to the genus bifidobacterium where they were affiliated with a high similarity of 99.8% to *B. breve* M4A and a 100% sequence identity to *B. longum* subps*. longum* FA1. The Phylogenic method from NCBI site was queried (figure 6). The accession numbers of the specific species of bifidobacteria were selected. The construction likelihood phylogeny tree was chosen and aligned using the MEGA 5.10 Program

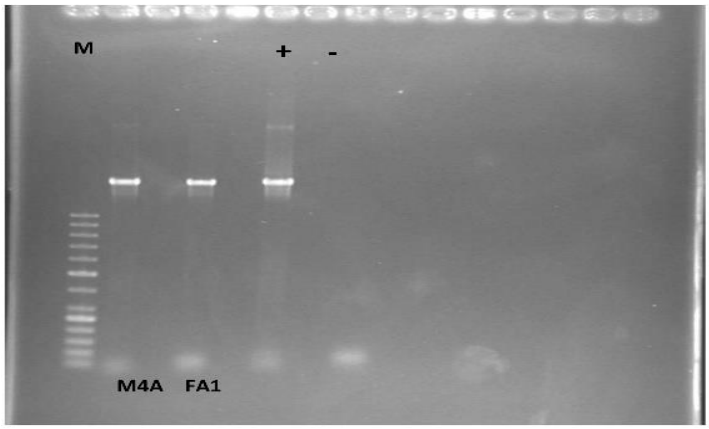


**Figure 5: Agarose-gel (1%) electrophoresis of PCR products. Lambda DNA/ EcoRI+ Hind III was used as marker (M). E. coli and water were used as positive and negative control, respectively. The (FA1) and (M4A) bands represented the amplified nucleotide sequences for B. longum subps. longum, and B. Breve, respectively.**

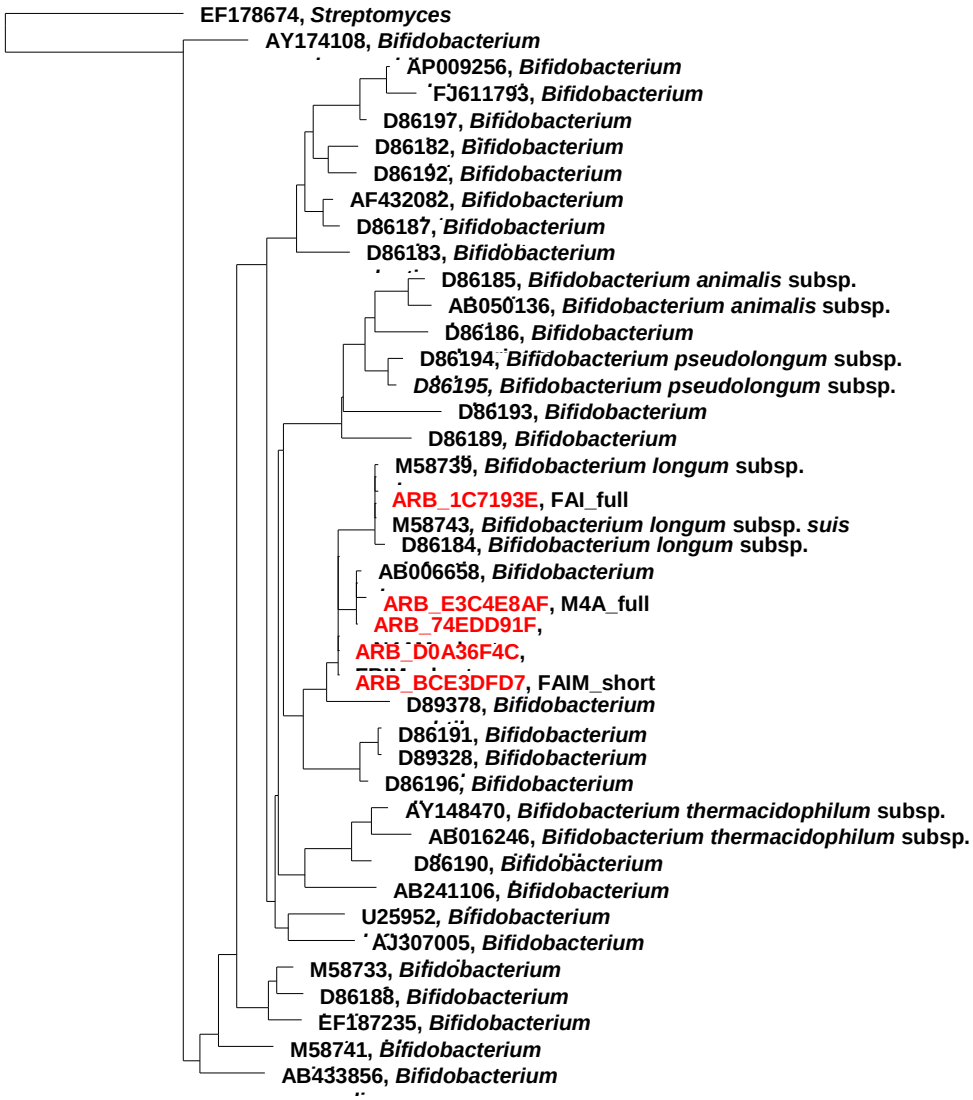


**Figure 6: Phylogenetic tree based on 16S rRNA gene sequences, showing the affiliation of the isolates of the present study *B. longum* subsp. *longum* FA1 and *B. breve* M4A with Bifidobacterium species.**

## 3.3. Biochemical tests and further characterization

Biochemical assays testing the organism's ability to utilize various saccharides were conducted because different species (strains) ferment different substrates and produce different enzymes epend on *Bifidobacteria* species (Holdeman *et al.* 1977). The biochemical tests were used to confirm the identification of *B. longum* subspecies. The carbohydrate fermentation patterns for *B. longum* subsp. *longum* are shown in Table 4.

They depend on their specific characteristics which have been identified by species-specific PCR with some similarities among *B. longum* sub-species (Palframan *et al.* 2003; Sakata *et al.* 2002).

Table 4: Carbohydrates fermentation by isolates of *Bifidobacterium longum*

| Isolate | Control | Glucose | L-Arabinose | Ribose |
|---|---|---|---|---|
| *B. longum* subsp*. Longum* FA1 | - | + | + | - |

## 3.4. Lyophilisation procedure

The CFU of the MRS broth culture before lyophilisation for *B. longum* subsp*. longum* FA1 and *B. breve* M4A was 6.77 and 6.87 log CFU/mL, respectively (Table 5). The survival rates were 71.8% and 81.5% for *B. longum* subsp*. longum* FA1 and *B. breve* M4A, respectively. The number of viable cell counts after six months storage were 5.06 and 6.84 log CFU/mL for *B. longum* subsp*. longum* FA1 and *B. breve* M4A, respectively. The survival rates after six months storage were 74.7% and 99.6% for *B. longum* subsp*. longum* FA1 and *B. breve* M4A*,* respectively.

Table 5: Viability of *B. longum* subps. *longum* FA1 and *B. breve* M4A before and after lyophilisation (log CFU/mL) with their survival rate.

| Culture | Viability before lyophilisation* | Viability after lyophilisation | Survival rate before Storage % | Viability after 6 months | Survival rate after storage % |
|---|---|---|---|---|---|
| *B. longum* subps. *longum* FA1 | 6.77 | 4.86 | 71.78 | 5.06 | 74.74 |
| *B. breve* M4A | 6.87 | 5.60 | 81.51 | 6.84 | 99.6 |

***** Lyophilisation at pH=4

## 3.5. pH-reduction

Figure 7 depicts continuous reduction of pH during a 48 h incubation period for *B. breve* M4A. The pH in the control was 4.71, whereas figure 8 shows that with all treatments pH decreased to less than 4. For *B. longum* subsp. *longum* FA1, the pH in the control was 5.03, whereas the pH with all treatments decreased to 4.

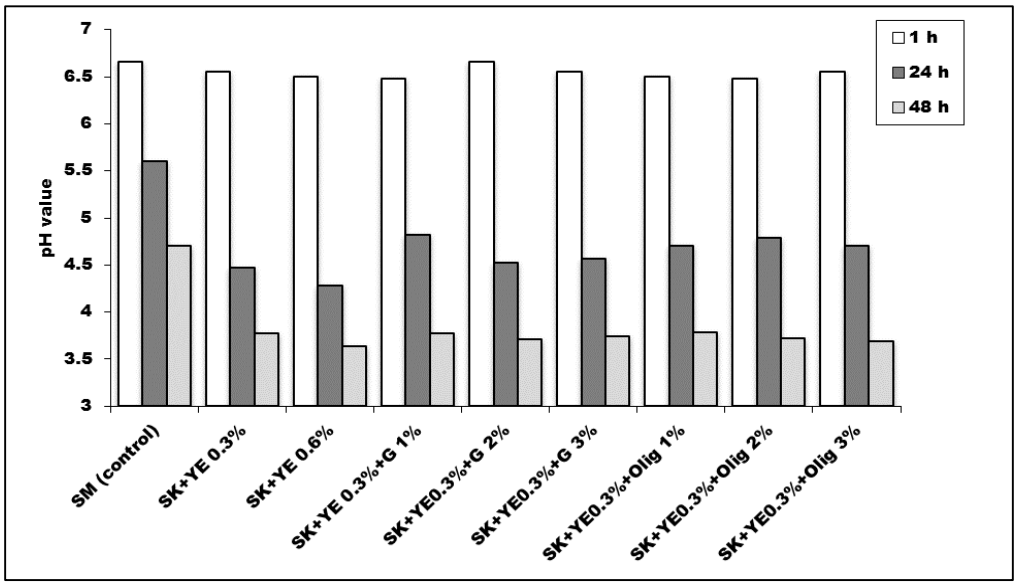


**Figure 7: Altered pH values of *B. breve* M4A after 48 h of incubation at 37˚C M4A in various supplemented milk media SK = skim milk media containing variations concentrations of YE = yeast extract, G = glucose, and Olig = oligofructose. Each histogram bar represents the mean value of duplicate.**

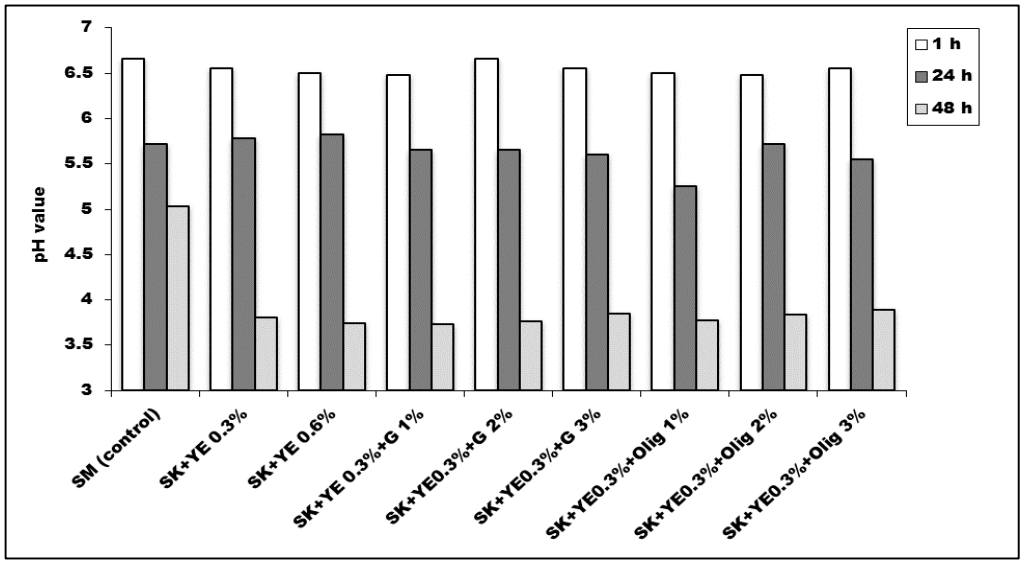


**Figure 8: Altered pH values of *B. longum* subsp. *longum* (FA1) after 48 h of incubation at 37˚C M4A in various supplemented milk media SK = skim milk media containing variations concentrations of YE = yeast extract, G = glucose, and Olig = oligofructose. Each histogram bar represents the mean value of duplicate.**

## 3.6. Use of supplemented media

Doubling time was used as a measure of the effects of yeast extract, glucose, and oligofructose concentrations on growth density of bifidobacteria. The enhancement of the growth rates of *B. longum* subsp*. longum* FA1 and *B. breve* M4A were evidenced by

significantly reduced doubling times compared to the controls. As depicted in figure (9) there was a dose-dependent growth improvement of *B. longum* subsp*. longum* FA1 secondary to 0.6% yeast extract and 0.3% yeast extract supplemented with either glucose or oligofructose at 1%, 2%, and 3%. Among the carbohydrate sources tested, treatment with skim milk + 0.3% yeast extract + 3% oligofructose was the most effective, resulting in a significantly reduced doubling time. All treatments involving supplementation with oligofructose resulted in a significantly decreased doubling time compared with treatments involved supplementation with glucose and yeast extract.

Figure (10) shows the enhancement of the growth of *B. breve* M4A by various supplements. Among the carbohydrate sources tested, skim milk + 0.3% yeast extract + 3% glucose provided the shortest mean doubling time and oligofructose resulted in a significantly reduced doubling time compared with treatment involving yeast extract as the sole supplement and skim milk + 0.3% yeast extract + 1% glucose.

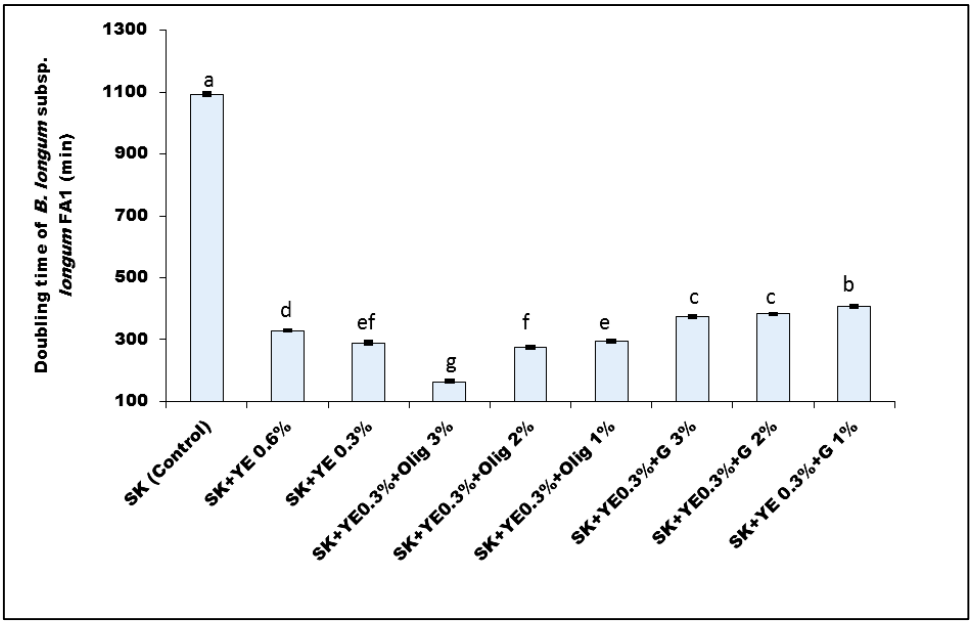


**Figure 9: Doubling time of *B. longum* subps. *longum* FA1 in SK=skim milk media containing different concentrations of YE = yeast extract, G = glucose, and Olig = oligofructose. Different letters indicates' highly significant (p<0.01) differences among treatment means (mean ±SD).**

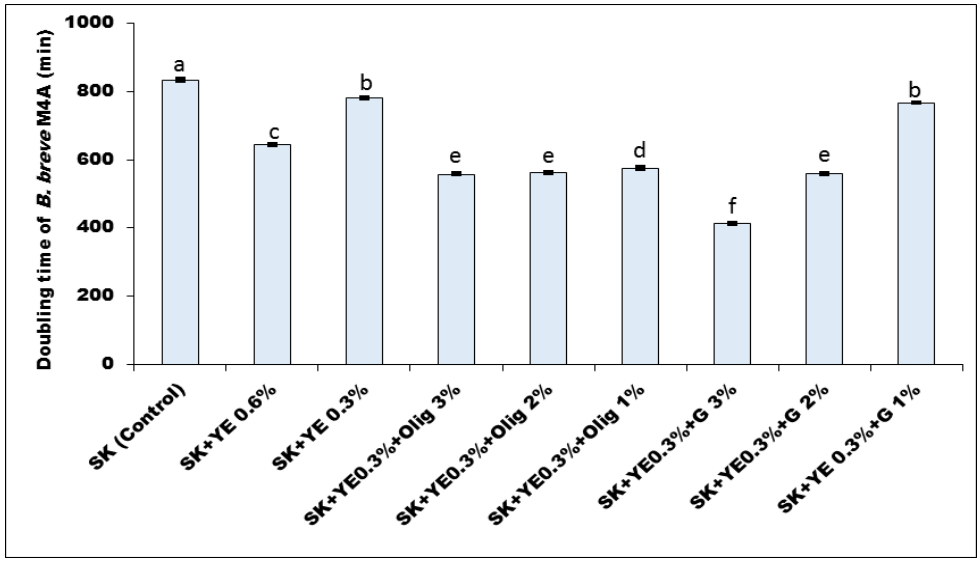


**Figure 10: Doubling time of *B. breve* M4A in SK = skim milk media containing different concentrations of YE = yeast extract, G = glucose, and Olig = oligofructose. Different letters indicates' highly significant ($p<0.01$) differences among treatment means (mean ±SD).**

## 3.7. Viability of cells

The highest viable cell number achieved by *B. longum subsp. longum* FA1 was 8.21 log CFU/ mL with the skim milk + 0.3% yeast extract + 1% oligofructose treatment and the lowest viable cell number was 7.70 log CFU/ mL for the skim milk (Control), followed by 8.11, 8.04, 8.03, 8.01, 7.96, 7.95 and 7.86 log CFU/ mL for the skim milk + 0.3% yeast extract + 3% glucose; skim milk + 0.3% yeast extract + 2% oligofructose; skim milk + 0.3% yeast extract + 3% oligofructose; skim milk + 0.6% yeast extract; skim milk + 0.3% yeast extract + 1% glucose; skim milk + 0.3% yeast extract + 2% glucose and skim milk + 0.3% yeast extract, treatments, respectively (Figure 11). The highest viable cell number achieved by *B. breve* M4A was 8.46 (log CFU/ mL) in the skim milk + 0.3% yeast extract + 2% oligofructose treatment and the lowest viable cell number was 8.17 for the skim milk (Control), followed by 8.43, 8.42, 8.42, 8.41, 8.40, 8.33 and 8.30 (log CFU/ mL) for the skim milk + 0.3% yeast extract + 3% oligofructose; skim milk + 0.6% yeast extract; skim milk + 0.3% yeast extract + 2% glucose; skim milk + 0.3% yeast extract + 1% oligofructose; skim milk + 0.3% yeast extract + 3% glucose; skim milk + 0.3% yeast extract and skim milk + 0.3% yeast extract + 1% glucose treatments, respectively.

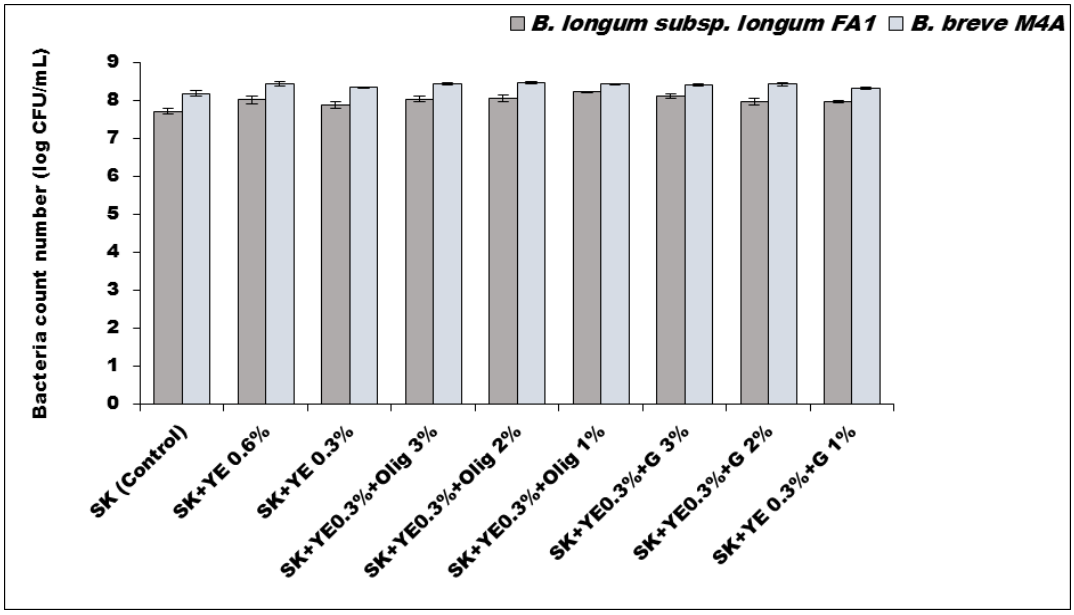


**Figure 11: Treatments with supplemented skim-milk media resulted in significant increases ($p < 0.01$) in viable cell numbers of both *B. breve* M4A and *B. longum* subsp. *longum* FA1 compared with skim-milk medium alone (control). SK=Skim milk; YE=Yeast extract; G= glucose; Olig= Oligofructose (mean ±SD).**

## 3.8. Surface response to the supplemented media

The response surface plots in figure (12) showed the relationship between the count number (log CFU/mL) of *B. longum* subsp. *longum* FA1 and different media. The maximum count number 8.21 and 7.70 (log CFU/mL) of *B. longum* subsp. *longum* FA1 were obtained from 3% glucose followed by 1% oligofructose supplemented medium, respectively.

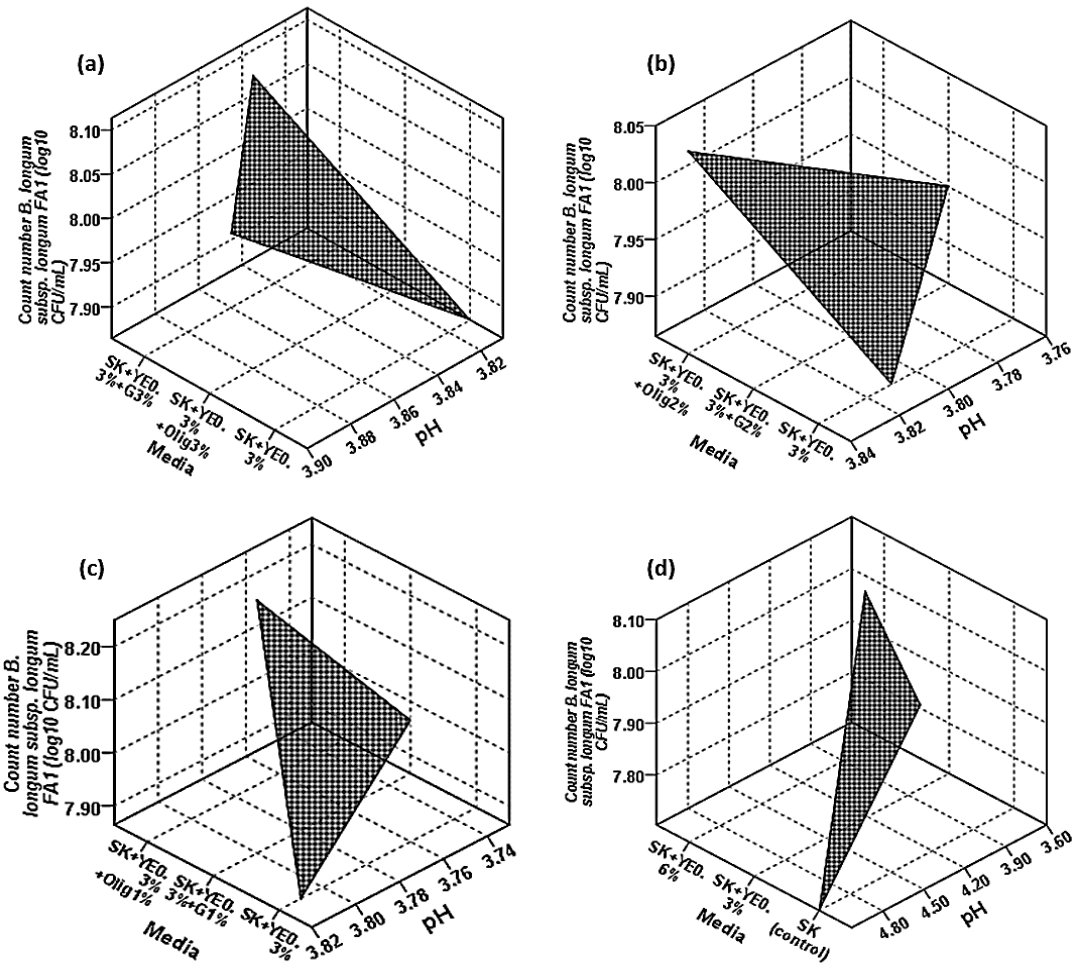


**Figure 12: Response surface plots for the count number (log CFU/mL) of *B. longum* subsp. *longum* FA1 with different media. The response surface factors are; (a) SK+YE0.3+Olig1%, SK+YE0.3+G1% and SK+YE0.3% media, and the pH. (b) SK+YE0.3+Olig2%, SK+YE0.3+G2% and SK+YE0.3% media, and the factor the pH. (c) SK+YE0.3+Olig3%, SK+YE0.3+G3% and SK+YE0.3% media, and the factor the pH. (d) SK, SK+YE0.3% and SK+YE0.6% media. (SK; Skimmed milk, YE; yeast extract, G; glucose, Olig; oligofructose).**

The highest acid production and the highest count number of the surface plot shown in figure (12a) whereas the lowest levels of acid production and count number of the surface plot illustrated in figure 12 (b, c & d) were the media with lower carbohydrate concentration and without carbohydrates. Figure (13) showed response surface plot representing an optimized count number 8.46 and 8.43 (log CFU/mL) of *B. breve* M4A in figure (13a) attributed to the oligofructose 3% and glucose 3% supplemented media, respectively. The supplemented skimmed milk media with 1% and 2% of carbohydrates showed in figure 13 (b, c & d) had been less improvement of the pH values as well as count number.

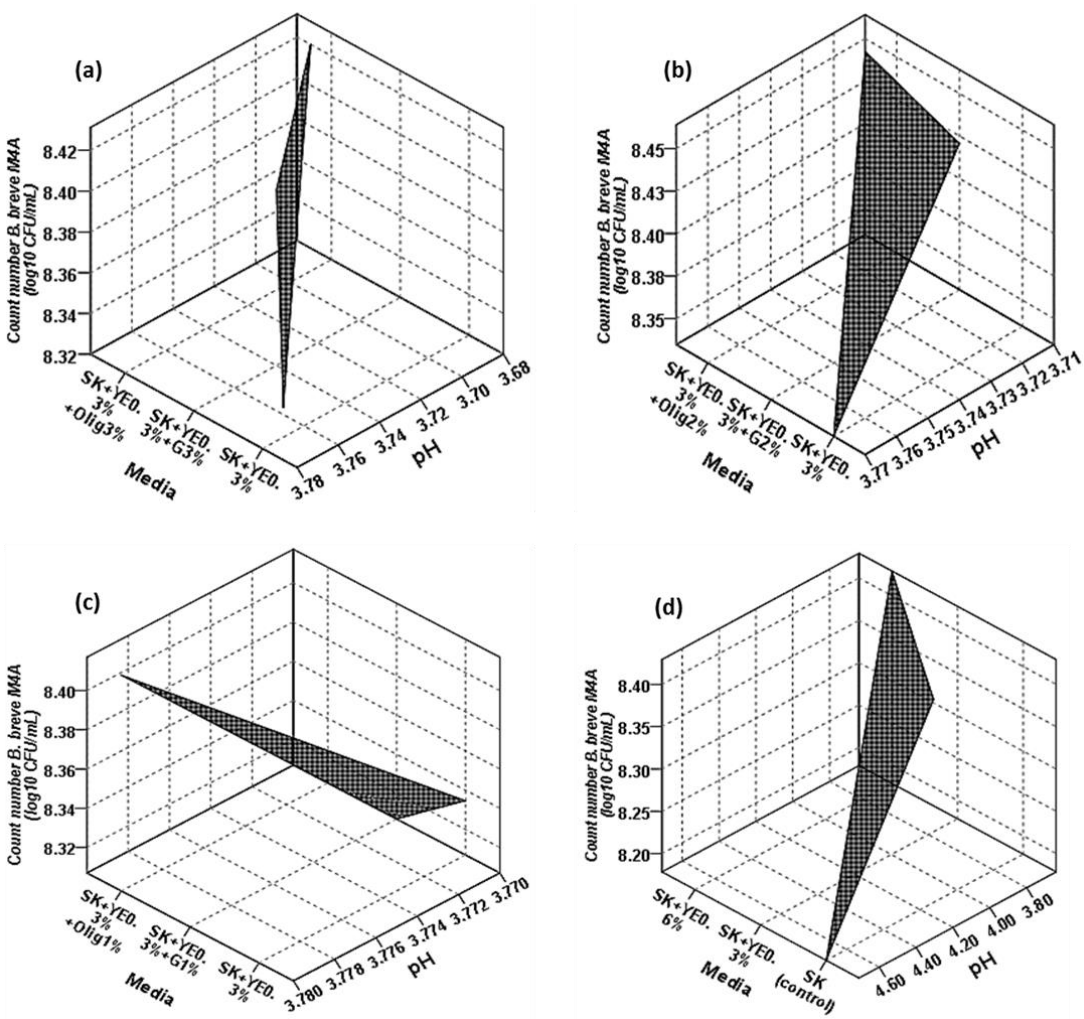


**Figure 13: Response surface plots for the count number (log CFU/mL) of *B. breve* M4A with different media. The response surface factors are; (a) SK+YE0.3+Olig1%, SK+YE0.3+G1% and SK+YE0.3% media, and the factor the pH. (b) SK+YE0.3+Olig2%, SK+YE0.3+G2% and SK+YE0.3% media, and the pH. (c) SK+YE0.3+Olig3%, SK+YE0.3+G3% and SK+YE0.3% media, and the factor the pH. (d) SK, SK+YE0.3% and SK+YE0.6% media. (SK; Skimmed milk, YE; yeast extract, G; glucose, Olig; oligofructose).**

## 3.9. Food intake

The total food consumption of seven weeks was 266.9, 198.4 and 165.1 g per mouse in the HFD, HFD-FA1 and HFD-M4A groups, respectively (Figure 14). The consumed diet of HFD-FA1 and HFD-M4A groups was decreased highly significant ($p < 0.01$) in week 4 and decreased significant ($p < 0.01$) of HFD-M4A group in week 3 in comparison with HFD group. Nevertheless, the increase in total food intake in the HFD group was not significantly higher than the HFD-FA1 and HFD-M4A groups.

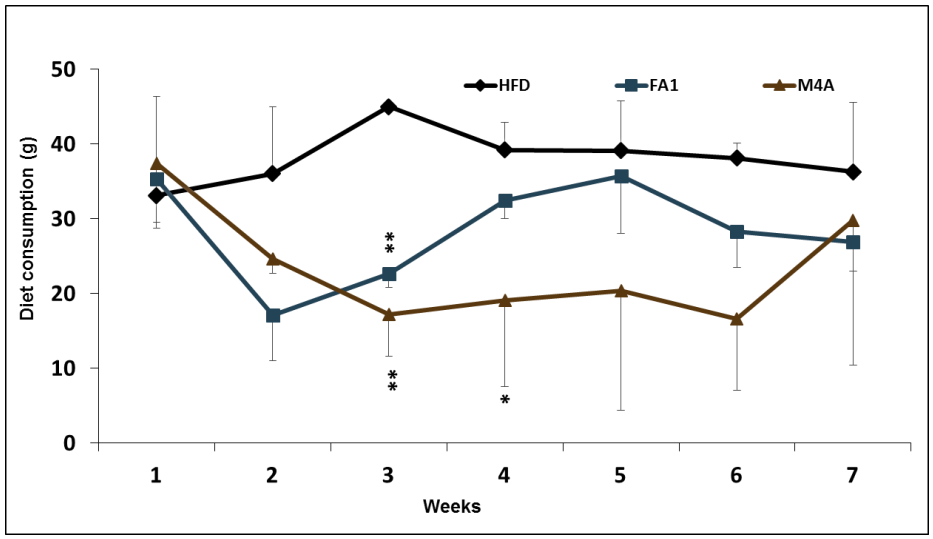


**Figure 14: Total diet consumption of mice treatments after 6 weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively (mean ±SD; * Significant at p<0.05; ** Significant at p<0.01).**

## 3.10. Body weight and weight gain

A significant difference (p<0.01) in body weight was already observed one week after the start of the supplementation phase between HFD-M4A group and HFD group, with a decreased weight gain in HFD-M4A group (figure 15).

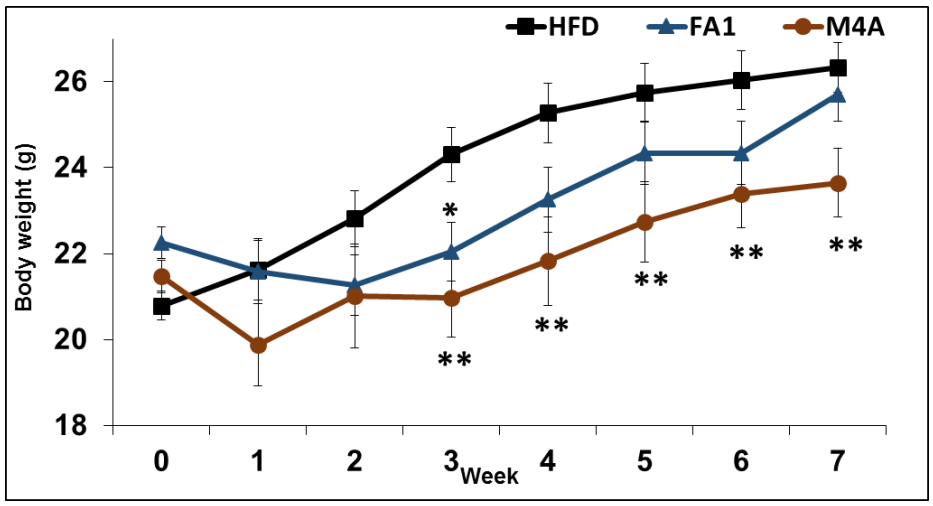


**Figure 15: Body weight development of the three treatment groups. One group received high-fat diet only, one received HFD and *B. breve* M4A (4.1×106 CFU/day), one received *B. longum* subsp. *longum* FA1 (2.9×106 CFU/day). From time 0 to 1, all groups were on control diet. HFD started from time point 1. Additional supplementation started at time point 2 and had a duration of six weeks. Data are expressed as mean ±SE; * Significant at (p<0.05); ** Significant at (p<0.01).**

The administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 decreased weight gain significantly ($p < 0.01$) in the HFD-M4A and HFD-FA1 groups compared with the HFD group that was 2.09, 3.44 and 5.54 g per mouse, respectively (Figure 16).

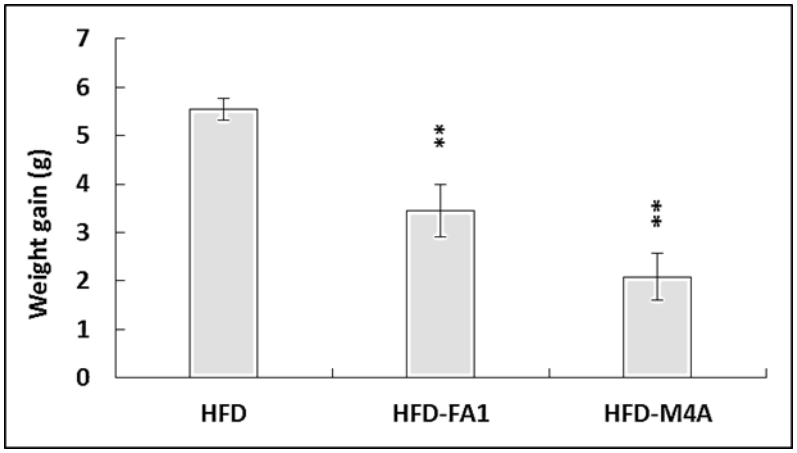


**Figure 16: Weight gain of mice after 6 weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. (mean ±SD; * Significant at p<0.05).**

## 3.11. Bacterial counts

The number of lactic acid bacteria in the intestines of the HFD-FA1 and HFD-M4A groups were 9.52 and 9.62 log CFU/g, respectively, which was increased highly significant ($p < 0.01$) in comparison with (6.68 log CFU/g) the HFD group (Figure 17).

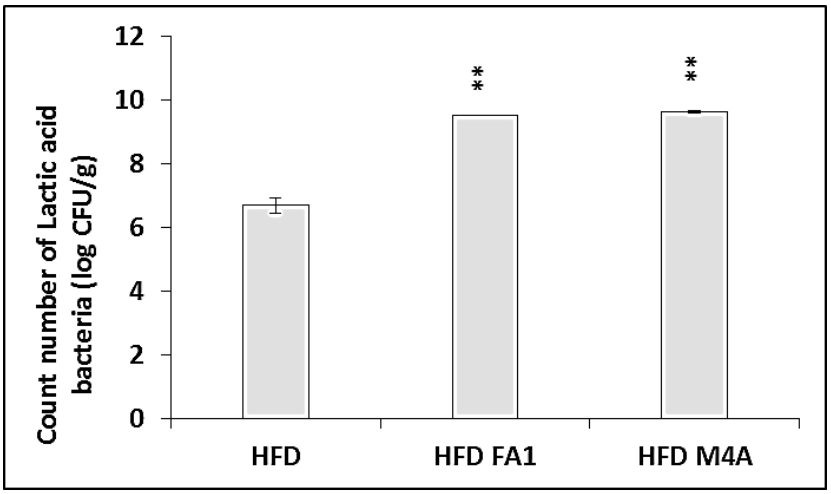


**Figure 17: Count number of lactic acid bacteria from intestinal contents of mice treatments after administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. (mean ±SE; ** Significant at p<0.01).**

The number of bifidobacteria increased highly significant ($p < 0.01$) in the HFD-M4A and HFD-FA1 groups was 8.80 and 8.76 log CFU/g, respectively, compared to 6.57 log CFU/g in the HFD group (Figure 18).

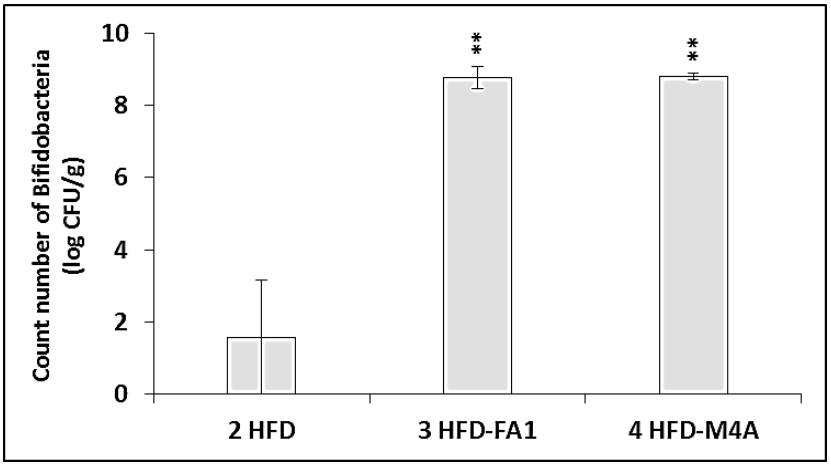


**Figure 18: Count number of bifidobacteria from intestinal contents of mice treatments after administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. (mean ±SE; ** Significant at p<0.01).**

## 3.12. Serum triglycerides

Figure (19) shows that the administration of *B. breve* M4A in the HFD-M4A group decreased triglyceride levels significantly ($p < 0.05$) to 125.38 (mg/dL) compared with 174.429 (mg/dL) in the HFD-group. There were no significant differences in triglycerides between (160.33 mg/dL) the HFD-FA1 and HFD groups. Total serum cholesterol levels and serum ALT enzyme levels (figure 20) were not significantly different among groups.

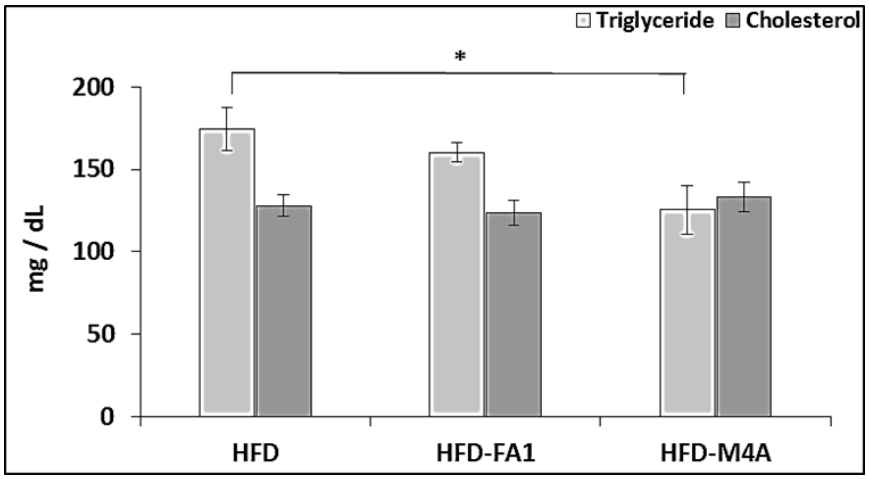


**Figure 19: Total cholesterol and triglyceride levels in serum of mice after six weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. (mean ± SE; * Significant at p<0.05)**

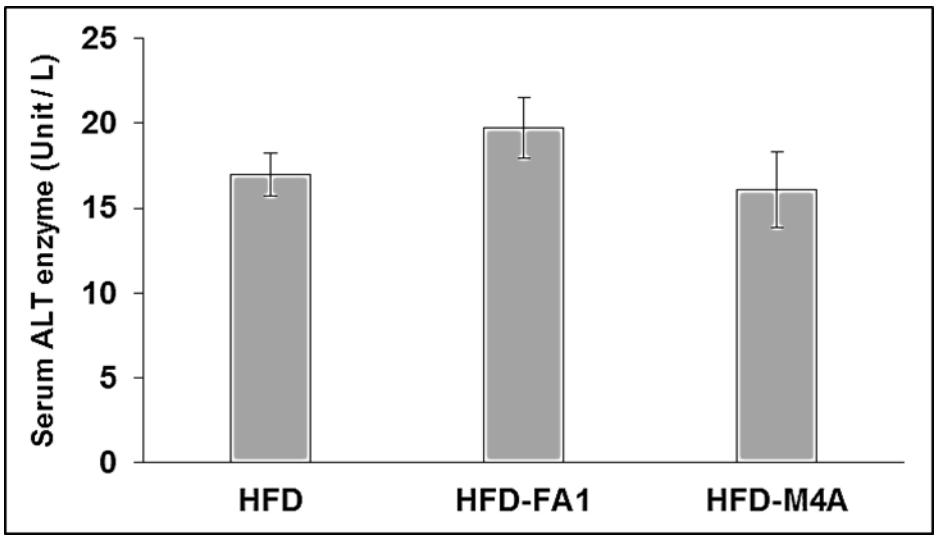


**Figure 20: Serum ALT enzyme level test (mean ± SE) of HFD-M4A and HFD-FA1 groups fed high fat diet in combination with *B. breve* M4A and *B. longum* subsp. *longum* FA1, respectively compared with HFD group.**

## 3.1. Hepatic triglycerides and cholesterol

The hepatic triglyceride content (figure 21) of the HFD-FA1 group was 12.8 mg/g and 11.1 mg/g in the HFD group, and 9.18 mg/g in the HFD-M4A group. However, there were no significant differences among group means of the triglyceride level. The liver cholesterol level was not significantly different among groups.

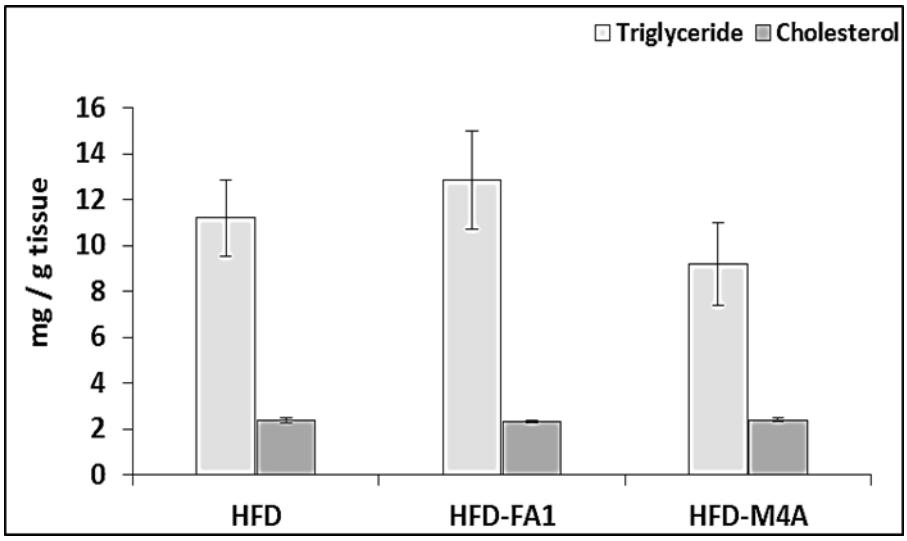


**Figure 21: Total cholesterol and triglyceride levels in liver of mice after six weeks of administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1 for HFD-M4A group and HFD-FA1 group, respectively. (mean ± SE).**

## 3.1. Bifidobacteria and hepatic histology

The Oil Red O-stained area was calculated to assess triglyceride accumulation in the liver using Image J software. The proportion of the lipid droplets area in cryosection images was 12.43% in the HFD group, which was significantly high ($p < 0.01$) compared with 3.40 and 2.46% in the HFD-FA1 and HFD-M4A groups, respectively (Figure 22).

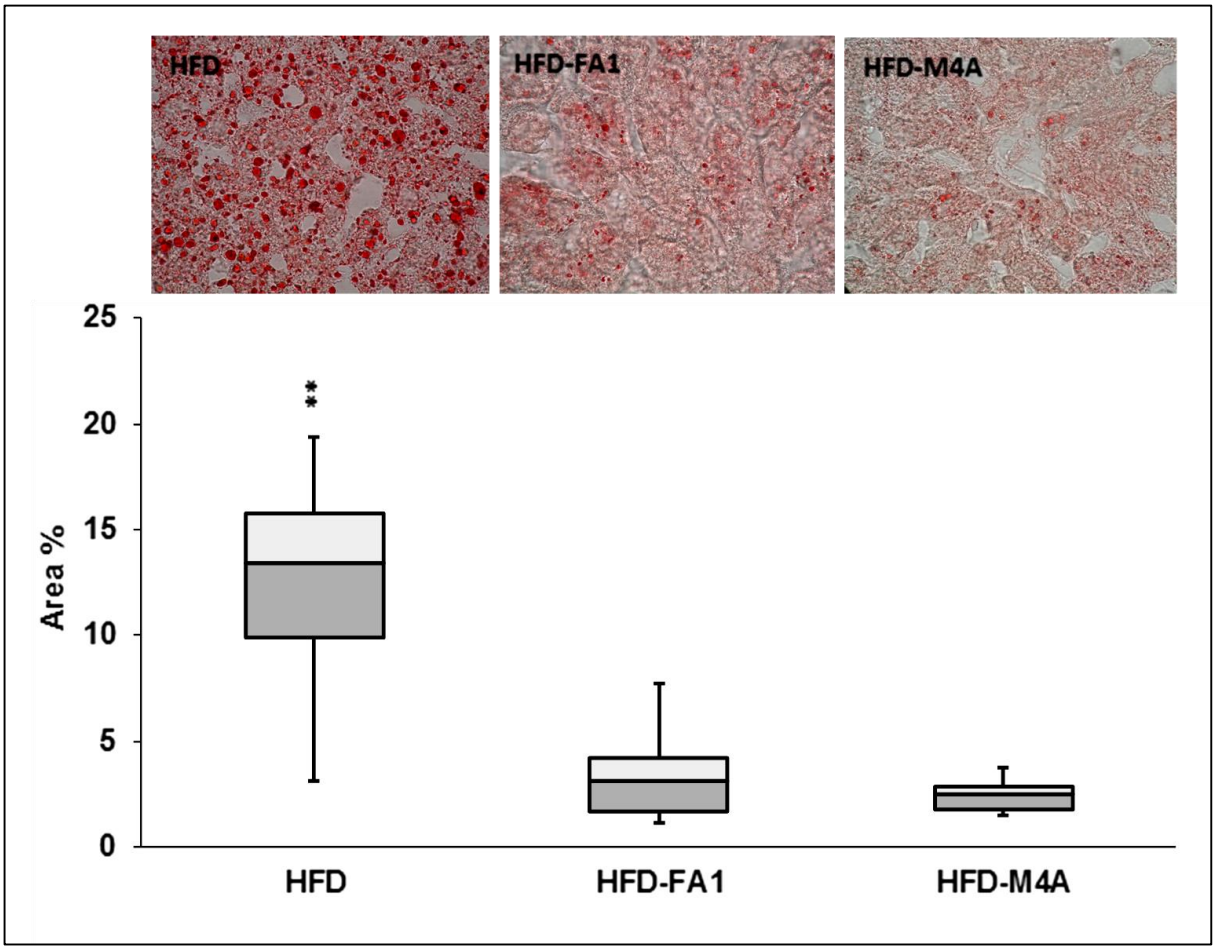


**Figure 22: Calculated area (%) of Oil Red O staining photos imaging cryosections to determine triglyceride accumulation in liver by Image J program. A: high fat diet; B: high fat-diet+*B. longum* subsp. *longum* FA1; C: high-fat diet+*B. Breve* M4A. Photos A, Photos A, B and C were Oil Red stained native cryo-lices/liver. Magnif. 200x and 1000x, bars 200µm. (mean ±SE; ** Significant at p<0.05).**

Figure (23) shows images of haematoxylin and eosin-stained liver sections (100× and 1000× magnification). The haematoxylin and eosin-stained section showed cells were being sized with fat content (figure 23 A). The different diets did not influence liver architecture or inflammatory status of the liver. The liver mass (figure 24) of mice was decreased significantly ($p<0.01$) by feeding with high fat diet in combination with *B.*

*breve* M4A to 0.90 g of the HFD-M4A group in comparison to 1.11 g of the HFD group. Whereas, no significant difference was demonstrated between the HFD group and (1.06 g) the HFD-FA1 group.

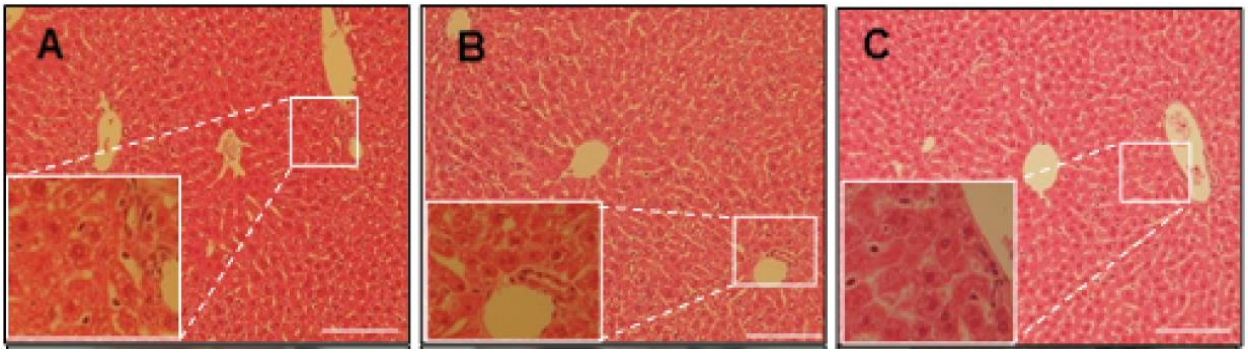


**Figure 23: Haematoxylin & eosin-stained paraffin slices of liver showing the microvascular lipid droplets. Magnification 100x and 1000x, bars 100µm. A: high fat diet; B: high fat-diet + *B. longum* subsp. *longum* FA1; C: high-fat diet + *B. Breve* M4A.**

The administration of *B. longum* subsp. *longum* FA1 and *B. breve* M4A to the HFD-FA1 and HFD-M4A groups, respectively, altered liver lipid droplet accumulation compared with the HFD group.

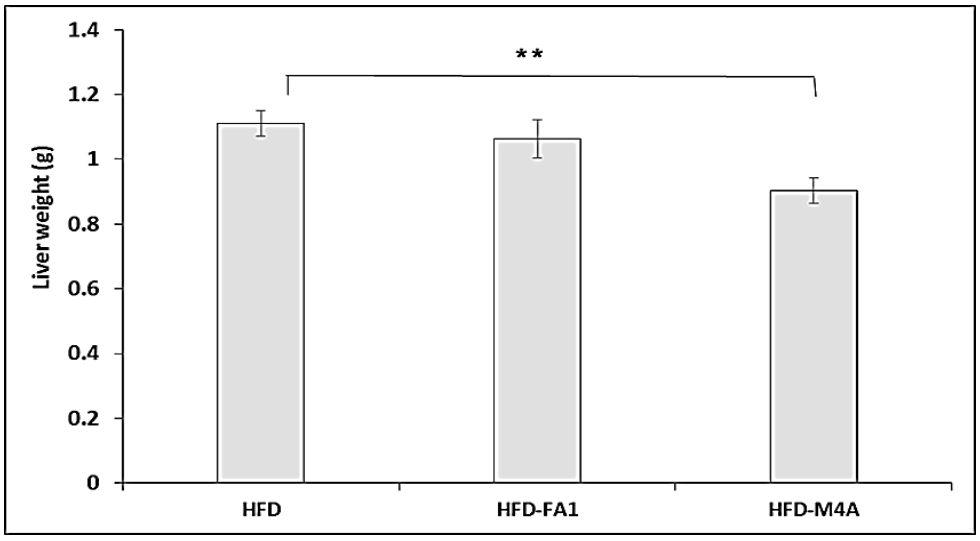


**Figure 24: Liver weight of mice after feeding with high fat diet in combination with B. breve M4A of HFD-M4A group. (mean ± SE). **highly significant (p<0.01).**

## 4. Discussion

Bifidobacteria are used as probiotics for the prevention and treatment of pathologies typical of newborns, such as necrotizing enterocolitis and streptococcal infections. Furthermore, allergic diseases, celiac disease, obesity, and neurologic diseases potentially presented new opportunities for probiotic applications of bifidobacteria (Di Gioia et al, 2014). Short chain fatty acids are the primary end products of carbohydrate metabolism in bifidobacteria lowering the pH and reducing the growth of undesirable microorganisms in the intestine, as well as to increase peristalsis pushing the gut contents forward and eventually helping to remove pathogens (Matsuki *et al.* 2013). Due to acetate production, *B. longum* subsp*. longum* strain JCM 1217T prevented the apoptosis of intestinal epithelial cells when *E. coli* O157 were administrated to germ-free mice (Fukuda *et al.* 2011).

Mupirocin MRS selective media was used for isolating *Bifidobacteria ssp.* from breastfed infant's faeces. The isolation and differentiation of bifidobacteria from other anaerobic bacteria can be difficult because of shared biochemical properties and habitats. The MRS mupirocin-based media appeared to suit best for enumeration of bifidobacteria in selective media (Simpson *et al.* 2003). Fortunately, bifidobacteria resist inhibition of the antibiotic mupirocin which may serve as a simple criterion distinguishing *Bifidobacterium spp.* from others (Rada 1997). With the lowest concentration of mupirocin it was found that bifidobacteria were outgrown by other bacteria while the concentration of 40 (mg/L) mupirocin suppressed the growth of most other bacteria (Figure 2). Previous studies have found that the addition of 25 (mg/L) mupirocin to transoligosaccharide propionate (TOS) agar medium resulted in inconsistent selectivity against non-bifidobacteria species of poultry caeca origin. The addition of 50 mg/l mupirocin to TOS makes it an effective selective medium for the isolation and enumeration of *Bifidobacterium spp*. from chicken faeca samples (Thitaram *et al.* 2005). As Figure 6 (d) shows, in this study the highest concentration of mupirocin (80 mg/L) gave the highest selectivity for the growth of bifidobacteria. While the addition of mupirocin at levels as high as 100 (mg/L) suppressed the growth of lactic acid bacteria, it still permitted the growth of *Bifidobacterium spp.* on broth pure cultures (Rada *et al.* 2000). Such findings indicate that most, if not all, bifidobacteria exhibit stable resistance to mupirocin at moderate-to-high levels 1,800–2,000(µg/ml) respectively (Serafini *et al.* 2011).

For this study numbers of colony-forming units (CFUs) have been determined from faeces of breast-fed infants. CFU of bacteria from a newborn (one day old) were non-detectable because the gastrointestinal tract is sterile at birth (Heavey *et al.* 1999) and becomes colonized by different microorganisms soon after birth (Mitsuoka 1996). Bifidobacteria are normally considered the most prevalent organism in the infant's gut (Franck 2007). In addition, Enterobacteria and Bifidobacteria represent early colonizers in breast-fed infants (Mountzouris *et al.* 2002).

Table 1 illustrates that the CFUs increased with the infants growing older, by which time the commensal bacteria start to develop a normal intestinal microbiota. The Bifidobacteria almost dominate the gut of breast-fed infants (Palmer *et al.* 2007; Saccaro *et al.* 2011).

The results presented in Figure 3 show a significant increase in bifidobacterial colonization in the infant with aging. This is because of the ingestion of bifidogenic factors in breastmilk that selectively stimulate the growth of Bifidobacteria (Collins *et al.* 1999; Tuohy *et al.* 2003). The highest count described in four-month-old infants were 8.47 and 8.79 CFUs while the count at 10 months was reduced to 7.93 CFUs because of the development of an ecosystem that resembles the adult profile (Mackie *et al.* 1999). This change is caused by the change of diet, weaning from breastmilk, physiological changes in the growing infant and gastrointestinal tract maturation (Hopkins *et al.* 2005).

Identification based on phenotypic traits does not always provide clear-cut results, therefore, detection of Bifidobacteria was achieved using PCR amplification and sequence analysis of the 16S rRNA gene (Figure 3). Identification based on the PCR technique was rapid and accurate for *B. longum* subsp*. longum* FA1 and *B. breve* M4A the same as those previously found in breastfed infants. *B. breve* was the most frequently detected species, followed by *B. infantis*, *B. longum* and *B. bifidum* when species-specific primers for bifidobacteria identification were used (Matsuki *et al.* 2003). *B. longum* subsp. *longum* FA1 was identified among different subspecies by carbohydrate fermentation test. A previous study had found that a *Bifidobacterium longum* strain had a higher survival rate when incubated in artificial gastric juice at pH 3.0 for 1.5 to two hours (Xiao *et al.* 2003).

In the present study, two strains of bifidobacteria were used because of their origin from the human microbiota of breastfed infants. Since several observations have been reported with *B. longum* and *B. breve* both strains were studied. The isolates were chosen for their higher viability as recommended for probiotic microorganisms (Fukuda *et al.* 2011; Vitali *et al.* 2007).

The viability of bifidobacteria in conventional media is limited. For long-term preservation, lyophilisation is the classical way of conservation and was also used in animal experiments (Heckly 1985). The detection of viable cell counts of *B. breve* M4A and *B. longum* subsp*. longum* FA1 following lyophilisation and conservation for six months suggested acceptable survival rates. Skim-milk fermentation increased the potential for acid production and decreased the doubling time in the presence of yeast extract, glucose, and oligofructose. Skim-milk media supplemented with yeast extract and glucose or oligofructose were effective in improving the growth of *B. breve* M4A and *B. longum* subsp*. longum* FA1, depending on the carbohydrate concentration and Bifidobacterium strain used. Bărăscu and coworkers monitored the growth rate of bifidobacteria in milk supplemented with yeast extract after 24 hours of incubation time. The number of cells in the presence of 0.1, 0.2, 0.3, and 0.4% yeast extract increased stepwise as compared to the cells grown in basal medium. The highest CFU count of bifidobacteria was obtained with basal medium plus 0.4% yeast extract (32 x $10^8$ CFU/$cm^3$). This was four times higher than that with basal medium 8 x $10^8$ CFU/$cm^3$ (Bărăscu *et al.* 2007).

Various studies have reported that the viability of bifidobacteria is often low in fermented dairy products (Adhikari *et al.* 2000). Dairy products, particularly fermented milk, are considered the best vehicle for delivering bifidobacteria to humans. Milk is a nutritional medium for promoting the growth of bifidobacteria, amino acids and low molecular weight peptides are present at low concentrations in milk only. Therefore, the bifidobacteria cultures exhibit reduced growth in milk. Many approaches have been used to promote the development of various bifidobacteria for accelerating milk fermentation. The ability of organisms to grow well in milk depends on their ability to metabolize milk proteins and lactose, both varying considerably among different strains (Ostlie *et al.* 2003). Published reports on using supplemented milk media with sugar and growth factors have been limited to laboratory-scale studies that were mainly used for sub-

culturing or propagating inoculums. In addition, no justifications were given for the concentrations used. Moreover, the formulations of industrial media products are rarely revealed because of the company's competitive advantage. Regulatory authorities worldwide are seeking assurance that probiotic products could deliver viable starter organisms in sufficient numbers to the intestine in order to provide a benefit to consumers (Stephenei *et al.* 2007).

Different levels of supplemented skimmed-milk media were studied for the growth of bifidobacteria and their acid production. The mediation analysis of skimmed-milk media supplemented with yeast extract, glucose and oligofructose showed an influence on the cell count and pH. The relationship was evaluated using supplemented media at different pH-levels via cell count as a mediator using multiple, nonlinear regression models. The design did not allow the study of interaction effects in such a model as in the Plakett-Burman design (Kiviharju *et al.* 2005). The response on surface plots showed that the maximum count of *B. longum* subsp. *longum* FA1 and *B. breve* M4A was achieved after 48 hours at a pH of four. For every increase in the concentration level of the carbohydrates, the number count increased with both *B. longum* subsp. *longum* FA1 and *breve* M4A, whereas the yeast extract levels alone exerted less influence on the cell count. The optimal growth of *B. longum* subsp. *longum* FA1 was obtained from skimmed-milk media supplemented with 3% glucose, while skimmed-milk media supplemented with 3% oligofructose showed a more efficiently optimized growth of *B. breve* M4A. This result indicates that the interactions between skimmed milk supplemented with yeast extract, and glucose or oligofructose may have a stronger influence on the growth rate of bifidobacteria. Consequently, the use of 3% carbohydrate concentration in skimmed-milk media significantly improves the pH and the cell count.

In the animal experiment food consumption was recorded throughout the seven-week study period. Mice fed a high-fat diet and treated with *B. breve* M4A and *B. longum* subsp. *longum* FA1 consumed a non-significantly different amount of food than other groups. Administration of *B. breve* M4A reduced total body weight gain compared to HFD only. Casas *et al.* (2000) revealed that *Lactobacillus reuteri* improved the intestine's ability to absorb and process nutrients and increase food conversion. Another study reported that *Lactobacillus reuteri* was the only species present at higher levels in the gut microbiota of obese individuals whereas levels of *B. animalis* and

*Methanobrevibacter smithii* were higher in non-obese subjects (Million *et al.* 2012). A high-fat diet is considered one of the environmental factors that contributes most to the epidemic of obesity (Peters 2003; Bray *et al.* 2004).

In the current study, mice fed a high-fat diet (HFD) had an increased body weight. The influence of six weeks of exposure to the HFD on weight gain was a positive fat balance. The gut microbiota produces energy from undigested food (Cani *et al.* 2009). The administration of *B. breve* M4A might modulate the composition of the gut microbiota in the regulation of energy homeostasis. Another variable that might influence weight gain was observed in the HFD-FA1 and HFD-M4A groups. *B. breve* M4A and *B. longum* subsp. *longum* FA1 may lead to an altered intestinal microbiota that minimizes the amount of energy stored in adipose tissue, thereby, reducing weight gain. The proposed mechanism was that gut microbiota play an important role in energy homeostasis in the host's intestine (Cani *et al.* 2009). Thus, weight gain was less in these two intervention groups. *B. breve* M4A and *B. longum* subsp. *longum* FA1 might reshape the balance of the intestinal microbiota and modulate body weight.

Each *B. breve* strain may have a different capability of carbohydrate utilization as another study suggested. The genetic accessibility and murine colonization capacity of this strain makes it a valuable model for understanding bifidobacterial–host interactions in the gut (O'Connell Motherway *et al.* 2011). Maximal counts and generation times of *B. breve, B. bifidum, B. infantis,* and *B. longum* were not influenced by 0.5% fructooligosaccharides(FOS) when the organisms were grown in infant formula (Dubey et al. 1996), but the group did not investigate FOS-concentrations above 0.5%. Consistent with these findings, (Shin *et al.* 2000) observed significant differences in mean doubling times when the bifidobacteria strain was grown in the presence of 0.5% FOS. Through the valuable content of amino acids, the yeast extract may contribute to the growth stimulation of bifidobacteria and also to improved viability in dairy products (Bărăscu *et al.* 2007).

The mean triglyceride level (125.3 mg/dL) in the HFD-M4A group was reduced significantly in comparison to the HFD group. However, the triglyceride concentration in HFD-fed mice (174.4 mg/dL) was above the normal range (71–164 mg/dL) in adult mice (Suckow 2001). The administration of *B. breve* M4A reduced plasma triglycerides to the normal range. This result is in line with a previous study which demonstrated lower

serum and liver triglyceride levels in a murine obesity model after treatment with different *Bifidobacterium spp*. strains. *Bifidobacteria breve* M13-4 *was associated with* a significant increase in body weight while *Bifidobacteria breve* L66-5 *resulted* in a decrease in body weight based on similar energy consumption (Yin *et al.* 2010).

The high-fat diet used in the current study contained 44% crude fat with the same proportion of cholesterol as the control diet; therefore, there was no significant difference in serum cholesterol among the groups. In a previous study, the administration of $10^8$ or $10^9$ (CFU/day) *B. breve* B3 in six-week-old mice for eight weeks suppressed body weight gain and epididymal fat, and reduced serum cholesterol (Kondo *et al.* 2010). This is in line with our data even though it occurred under different conditions—e.g. age of mice, diet composition, and time of treatment.

Mice fed a HFD exhibited an altered composition of the gut microbiota and different levels of lactic acid bacteria. The administration of *B. longum* subsp*. longum* FA1 and *B. breve* M4A increased the proportion of lactic acid bacteria and bifidobacteria. These species contribute to a beneficial intestinal microbiota (Venema *et al.* 2003). The number of bifidobacteria was found inversely correlated with fat mass development by others already (Casas *et al.* 2000).

The dose of bacteria was chosen depending on the ability of bifidobacteria to grow in milk aiming at the number of viable cells required to meet the probiotic definition criteria. Mice in the intervention groups were given a daily oral dose ($10^6$ CFU) of viable cells in 0.2 mL, the maximum probiotic fermented milk volume that can be administered to mice (Minelli *et al.* 2008). The doses of bifidobacteria were increased in milk-supplemented media as much as possible. For achieving the beneficial effects of probiotics scientists have proposed that the product should contain probiotic bacteria with a minimum viable number of $10^6$ CFU/mL and recommended $10^8$ CFU/mL or per gram (Sukumar 2013; Minelli *et al.* 2008). Various authors have reported a low viability of Bifidobacteria in fermented dairy foods (Adhikari *et al.* 2000).The ability of organisms to grow well in milk depends on their ability to metabolize milk protein and lactose, and this ability varies considerably among strains (Ostlie *et al.* 2003).

Studies in humans have confirmed oligofructose's ability to increase the proportion of bifidobacteria in stools (Gibson *et al.* 1995). The growth of bifidobacteria showed improvement after the addition of (0.1 g/day per mouse) oligofructose in the drinking

water of both the HFD-FA1 and HFD-M4A groups. Oligofructose was applied to aid bacteria adherence in the mice's intestine as the strains were of human origin. The dosage and duration of supplementation with oligofructose in the present study was lower, i.e. 0.1 g/day for six weeks compared to another study which used 0.4 g/day or higher for at least eight weeks (Delzenne *et al.* 2002). Thus, it is unlikely that the increase in bifidobcteria was due to oligofructose.

The main histological characteristic of non-alcoholic fatty liver diseases is the accumulation of fat in the form of triglycerides in liver cells (Brunt 2010). The triglyceride content was measured in liver samples to investigate whether the bifidobacteria strains (*B. longum subsp. longum* FA1 and *B. breve* M4A) reduced hepatic fat accumulation and, consequently, liver weight. In the current study, only *B. breve* M4A reduced liver weight. A reduction of lipid droptlet leads to a lower risk of metabolic diseases (Greenberg *et al.* 2011).

The accumulation of lipid droplets in Oil Red O-stained images of liver sections was decreased by the administration of *B. breve* M4A and *B. longum* subsp. *longum* FA1. Haematoxylin and eosin-stained images of hepatic tissue from the HFD-fed mice group revealed a progressive increase in steatosis. (Lee *et al.* 2013) evaluated the effects of fermented lotus extracts on inhibiting differentiation in 3T3-L1 pre-adipocytes. Different probiotics, including *L plantarum*, *L rhamnosus*, *B. breve*, and *B. longum,* were used separately. In their study the inhibition of pre-adipocyte development was examined using Oil red O dye staining and all groups fermented by four kinds of probiotics showed reduction in triglyceride deposition.

In obese animals and humans triglyceride storage in adipocytes is increased. The elevated number of lipid droplets in skeletal muscle and liver cells causes hyperlipidemia (Greenberg *et al.* 2011; Lee *et al.* 2013). In the present study the hepatic mass of mice fed a high-fat diet was negatively correlated with the administration of bifidobacteria. Thus, oral supplementation with *B. breve* M4A attenuates hepatic fat accumulation.

The quantitative analysis of liver triglycerides did not correlate to the histological outcome of lipid staining of the microscopic slides from liver samples. As the calculation of the sample size was based on weight gain and not on the liver triglyceride levels, a significant reduction may be observed with an higher sample sizes in future studies.

Dhiman *et al.* (2014) assessed the efficacy of a probiotic preparation that could prevent the recurrence of hepatic encephalopathy, reduce the number of hospitalizations, and diminish the severity among cirrhosis patients. The intervention used in their study comprised daily intake of probiotics consisting of *L. acidophilus, L. paracasei, L. delbrueckii* subsp. *bulgaricus, L. plantarum, B. breve, B. infantis, B. longum* and *Streptococcus thermophiles* for six months. The reduction was significant in the development of hepatic encephalopathy among patients receiving the probiotic in comparison to in the placebo group. The Child–Turcotte–Pugh and model for end-stage liver disease scores were used to assess the prognosis of chronic liver disease, mainly in patients with cirrhosis. At 24 weeks of the study, there was clinically significant progress in parameters among the patients treated with the probiotic which modulated the gut microbiota by altering numbers, composition, and functions of bacteria, finally resulting in a reduction of serum ammonia levels.

## 5. Conclusion

The isolation of bifidobacteria from infant faeces was best at 80 (mg/L) mupirocin in MRS media, and the shape of bifidobacteria cells was affected by mupirocin. The amount of bifidobacteria in the faeces of breast-fed infants increased with the progression of age of the infants.

The sequencing of the 16S rRNA gene was performed for identifying the *Bifidobacterium* species. For lyophilization the cultures were harvested at pH 4 in MRS broth media. The survival rates of bifidobacteria after lyophilization and subsequent storage for six months were acceptable. The optimized protocols for bifidobacteria fermentation and lyophilization might be helpful for upscaling and industrial production of bifidobacteria-supplemented food. Skimmed-milk fermentation increased the potential for acid production and decreased the doubling time in the presence of yeast extract, glucose, and oligofructose. Skimmed-milk media, supplemented with yeast extract and glucose or oligofructose, was effective in improving the growth of the two strains studied, depending on the carbohydrate concentration and the bifidobacterium strain used. The dietary supplementation with bifidobacteria may be helpful to reduce hepatic steatosis, weight gain, and elevated triglyceride serum concentrations under a high-fat diet.

## 6. Summary

A high prevalence of overweight and obesity has been observed in the past few decades in many countries. Overall, policies for production of healthier foods and environments are recommended for the prevention of obesity. Until recently, little action has been taken on this issue (Swinburn *et al.* 2013). It has been proposed that alterations in the composition of the gut microbiota known as dysbiosis may be related to the development of obesity (Neyrinck *et al.* 2012). The first goal of this study was to isolate the predominant *bifidobacterium* species from the stools of breast-fed infants, and to identify them by their 16S rRNA genes. The strains that were identified were *B. longum* subsp*. longum* FA1 and *B. breve* M4A. The survival rates measured six months after lyophilisation were 74.74% and 99.6% for *B. longum* subsp. *longum* FA1 and *B. breve* M4A, respectively.

In a second step the growth of *B. breve* M4A and *B. longum* subsp. *longum* FA1 was examined in skimmed-milk media supplemented with different levels of yeast extract and in combination with glucose or oligofructose. These media increased the count of the strains after conservation by lyophilisation. Supplementation with glucose or oligofructose decreased the doubling time and increased the viable cell count. Factorial design showed a nonlinear relationship between skimmed-milk media, bacterial count number and acid production indicating an opposite impact of yeast extract with the supplementation of glucose or oligofructose in skimmed-milk media.

Response surface plots were applied to optimize the supplemented skimmed-milk media. Both strains grew more rapidly in supplemented media than in the skimmed milk-based medium alone. The fermentation in skimmed milk was dependent on nutrient availability and the carbon source. The addition of yeast extract with glucose or oligofructose to skimmed milk increased the growth rate and acid production, compared with skimmed milk alone, when incubated anaerobically at 37°C for 48 hours. Acid production was higher in *B. breve* M4A than in *B. longum* subsp. *longum* FA1 when inoculated into skimmed milk supplemented with yeast extract with glucose or oligofructose.

The second goal of the study was to evaluate the *in vivo* anti-obesity effect of *B. breve* M4A and *B. longum* subsp. *longum* FA1 in young mice fed an high-fat diet (HFD). Three (male mice C57BL/6JRj) groups, the model HFD group and treatment (HFD-FA1 and

HFD-M4A) groups were fed an HFD to induce obesity. After feeding the mice a HFD for six weeks, animals receiving *B. breve* M4A ($4.1 \times 10^6$ CFU/mL) and *B. longum* subsp. *longum* FA1 ($2.9 \times 10^6$ CFU/mL) had significantly lower ($p < 0.01$) weight gain compared to mice fed a high-fat diet only. Mice fed *B. breve* M4A supplemented with 0.3% yeast extract and 3% glucose exhibited significantly lower serum triglycerides ($p < 0.05$) compared with the HFD group. The daily consumption ($2.9\times10^6$ CFU/day) of *B. longum* subps. *longum* FA1 and ($4.1\times10^6$ CFU/day) *B. breve* M4A ($p < 0.01$) significantly increased the amount of bifidobacteria and lactic acid bacteria in the large intestine.

This study showed that *Bifidobacterium* species and their acid-production reduced weight gain and energy metabolism. Thus, bifidobacteria supplementation may be one mean for reducing obesity and related chronic Non-commuicable diseases.

# 7. Zusammenfassung

In den vergangenen Jahrzehnten wurde in vielen Ländern eine steigende Prävalenz von Übergewicht und Adipositas beobachtet. Allgemein werden zur Adipositasprävention sowohl die Herstellung gesundheitsfördernder Lebensmittel als auch eine gesundheitsfördernde Umwelt empfohlen. Dennoch sind bisher wenige erfolgreiche Maßnahmen implementiert worden (Swinburn *et al.* 2013).

Es wurde vermutet, dass Veränderungen in der Zusammensetzung der Darmmikrobiota, bekannt als Dysbiose, mit der Entstehung von Adipositas zusammenhängen (Neyrinck *et al.* 2012).

Erstes Ziel der vorliegenden Studie war, vorherrschende Bifidobacterium-Arten aus dem Stuhl von gestillten Säuglingen zu isolieren und anhand ihrer 16S rRNA Gene zu identifizieren. Gefunden wurden *B. longum* subsp. *longum* FA1 und *B. breve* M4A. Die Überlebensrate der Bakterien, gemessen sechs Monate nach Lyophilisierung, lag bei 74.74% für *B. longum* subsp. *longum* FA1 beziehungsweise 99.6% für *B. breve* M4A.

Dann wurde das Wachstum von *B. breve* M4A und *B. longum* subsp. *longum* FA1 in Magermilch-Medien untersucht, die mit verschiedenen Konzentrationen von an Hefeextrakt in Kombination mit Glucose oder Oligofructose angereichert waren. Diese Medien erhöhten die Anzahl an Stämmen nach Konservierung mittels Lypophilisierung. Die Supplementation verkürzte die Verdopplungszeit und erhöhte die Anzahl der lebensfähigen Zellen. Der faktorielle Versuchsplan zeigte eine nicht lineare Beziehung zwischen Magermilch-Medien, Bakterienanzahl und Säureproduktion. Dies deutet auf eine gegensätzliche Auswirkung von Hefeextrakt mit Glucose- oder Oligofructose-Supplementation in Magermilch-Medien hin. Response Surface Plots wurden angewandt, um die supplementierten Magermilch-Medien zu optimieren. Beide Stämme wuchsen schneller in supplementierten Medien als in Medien auf Magermilchbasis alleine. Die Säureproduktion lag höher bei *B. breve* M4A als bei *B. longum* subsp. *longum* FA1, wenn sie in Magermilch supplementiert mit Hefeextrakt sowie Glucose oder Oligofructose inokuliert wurde. Die Fermentationsfähigkeit in Magermilch hing von Nährstoffverfügbarkeit und der Kohlenstoffquelle ab.

Weiteres Ziel der Studie war es, die Anti-Adipositas Aktivität von *B. breve* M4A und *B. longum* subsp. *longum* FA1 an jungen Mäusen, denen eine fettreiche Nahrung gefüttert wurde, zu untersuchen. Drei (Male mice C57BL/6JRj) Gruppen, einer Kontroll-HFD-Gruppe und beiden interventionsgruppen (HFD-FA1 und HFD-M4A) wurde eine fettreiche Nahrung gefüttert, um Adipositas hervorzurufen. Nach einem Zeitraum von sechs Wochen zeigten Mäuse, die *B. breve* M4A ($4.1 \times 10^6$ CFU/day) und *B. longum* subsp. *longum* FA1 ($2.9 \times 10^6$ CFU/day) erhalten hatten, einen statistisch signifikant niedrigeren ($p < 0.01$) Gewichtszuwachs im Vergleich zu Mäusen, die nur die fettreiche Nahrung erhielten. Mäuse, die mit B. breve M4A, welches mit 0.3% Hefeextrakt und 3% Glucose angereichert war, gefüttert wurden, wiesen signifikant niedrigere Serum Triglyceride ($p < 0.05$) auf im Vergleich zu der HFD Gruppe. Täglicher Konsum ($2.9\times10^6$ CFU/day) von *B. longum* subps. *longum* FA1 und ($4.1\times10^6$ CFU/day) *B. breve* M4A erhöhte signifikant ($p < 0.01$) die Anzahl an Bifidobakterien und zu den Milchsäurebakterien im Dickdarm.

Die Untersuchungen zeigen, dass die geringere Gewichtszunahme, die günstigeren und die geringere Fettspeicherung in der Leber von der Gabe der untersuchten Bifidobakterien und deren Säureproduktion günstig beeinflusst wurden. Demzufolge erscheint eine Anreicherung der Nahrung mit Solchen als förderlich, um Adipositas und damit zusammenhängende Gesundheitsfolgen zu vermeiden.

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