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Dose dependent anti-obesity effect of three different *Lactobacillus sakei* strains using a diet induced obese murine model

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ABSTRACT

Overweight and obesity are considered as a major cause of various conditions related to metabolic syndrome. Yet, considering the complex interacting factors leading to pathogenicity and underlying mechanisms, it remains a poorly defined area. Some probiotics have a reputation of a relatively long history of safe use, and an increasing number of studies are confirming benefits including anti-obesity effects when administered in adequate amounts. Recent reports demonstrate that probiotic functions may widely differ with reference to either intra-species or inter-species related data. Such differences do not necessarily reflect or explain strain specific functions of a probiotic, and thus require further assessment at the intra-species level. Various anti-obesity clinical trials with probiotics have shown discrepant results and require more consolidated studies in order to clarify the correct dose of application for reliable and constant efficacy over a long period. In this study three different strains of *Lactobacillus sakei* were administered in a high fat diet induced obese murine model using three different doses, $1x10^{10}$ CFU, $1x10^{9}$ CFU and $1x10^{8}$ CFU, respectively, per day. Changes in body and organ weight were monitored, and serum chemistry analysis was performed for monitoring obesity associated biomarkers. The results show that only one strain of *L. sakei* (CJLS03) induced a dose dependent anti-obesity effect, while no correlation with either dose or body and adipose tissue weight loss could be detected for the other two *L. sakei* strains (L338 and L446). The body weight reduction mainly correlated with adipose tissue and obesity associated serum biomarkers such as triglycerides. This study suggests that anti-obesity effects of probiotics may vary in a strain and dose specific manner.

Keywords: *Lactobacillus sakei*, probiotic, dose dependency, strain specificity, fat mass, obesity

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INTRODUCTION

Overweight and obesity result from abnormal adipose deposition and function and are considered as major pathophysiological symptoms of metabolic syndrome (*Olufadi & Byrne, 2008*). Originating from insulin resistance, metabolic syndrome may be reflected by several clinical manifestations such as atherosclerosis, hyperglycemia, dyslipidemia, hypertension, reduced high-density lipoprotein cholesterol and type 2 diabetes mellitus (*Furukawa et al., 2017*). Based on typical pathological symptoms, broadly defined as excessive fat mass in the body (specifically the abdomen), the prevalence of obesity has rapidly increased during the last two decades (*Kobyliak et al., 2017*). In spite of intensive research input in recent history, deeper understanding of pathogenesis and knowledge on the underlying mechanisms of obesity are still limited, while, in fact, the causality of obesity has been suggested from different viewpoints and disciplines of science such as genetics, endocrinology and psychology (*Schwartz et al., 2017*).

Following up on classical approaches, recent studies show that microbiota can play a key role in host obesity and metabolic syndrome (*Gérard, 2016*). Thereby, new clinical diagnostic perspectives were opened on the influence of gut microbiota on the status of metabolic disorders. Numerous studies have reported on qualitative and quantitative discrepancies in microbiota of the gastrointestinal tract (GIT) when comparing healthy subjects with people suffering from metabolic diseases (*Turnbaugh et al., 2006; Turnbaugh et al., 2008; Ley et al., 2005; Cani & Delzenne, 2009; Armougom et al., 2009*).

There is general consensus that probiotics support the host gut microbiota balance, and scientific evidences are steadily accumulating regarding the beneficial impact of probiotics on human health, including immune disorders, inflammatory bowel disease, type 2 diabetes and atherosclerosis (*Amar et al., 2011; Kim et al., 2016; Ritze et al., 2014; Schroeder et al., 2018; Vemuri, Gundamaraju & Eri, 2017*). Various modes of probiotic action were elucidated by using *in vitro* studies (including *in vitro* models) while efficacy was investigated by *in vivo* studies and clinical trials. Along with therapeutic benefits, anti-obesity effects of probiotics have been reported recently (*Kadooka et al., 2010; Park et al., 2016; Wang et al., 2015; Woodard et al.,*

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2009). Yet, the anti-obesity efficacy of probiotics has not been fully elucidated in spite of various clinical trials, and scientific evidence for a "minimal dose effect level" remains relatively sparse (*Tanentsapf, Heitmann & Adegboye, 2011*; *Raoult, 2009; Mekkes et al., 2013*). The concept of a minimal effective dose is complicated due to the large (and diverse) number of microbial and host-related factors (*Salminen et al., 1998*), and will also depend on the kind of key criteria and the "end-points" selected. The dose of intolerance is generally considered to be high, thus, allowing a relatively broad "therapeutic window" (*Collins, Thornton & Sullivan, 1998*), it may be difficult to find a suitably effective low dose above the minimal level. Yet, precisely defining an effective dose has remained an arbitrary issue, and thus the pragmatic suggestion by an FAO/WHO Working Group (*FAO/WHO, 2002*) that "The suggested serving size must deliver the effective dose of probiotics related to the health claim". Convincingly delivering this kind of evidence has remained difficult until this day, in particular for commercial distribution of (food or pharmaceutical) strains claimed to be probiotics. In an early report *Perdigón, Alvarez & de Ruiz Holgado (1991*) suggested a dose related impact of *Lactobacillus casei* on the secretory immune response and protective capacity in intestinal infections. A placebo-controlled study designed to evaluate the therapeutic value of four different non-antibiotic preparations (including *Saccharomyces boulardii*, and heat-killed microbial strains) indicated a non-significant dose dependency for either prophylaxis or treatment of traveller's diarrhoea (*Kollaritsch et al., 1989; Kollaritsch et al., 1993*). Yet, substantial evidence supports the principle of dose dependency of probiotics to modulate systemic and mucosal immune function, improve intestinal barrier function, alter gut microbiota, and exert metabolic effects on the host, also in a strain dependent manner (*Alemka et al., 2010; Madsen, 2012*). *Everard et al. (2011*) reported a dose-dependent immunomodulation of human DCs by the probiotic *Lactobacillus rhamnosus* Lcr35, leading, at high doses, to the semi-maturation of the cells and to a strong pro-inflammatory effect.

In this study we administered three different ten-fold doses of three different *L. sakei* strains separately to a diet induced obese C57BL/6 murine model and monitored body weight during the full experimental period. Organ weights and serum biomarkers were monitored to

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elucidate the dose dependent anti-obesity effect of three different *Lactobacillus sakei* strains.

MATERIALS AND METHODS

The animal study was approved by the Ethical Committee of KPC Ltd. in Korea (P150067). Five weeks old, specific pathogen free (SPF) male C57BL/6 mice were supplied from Orient Bio, Korea. High fat diet (Research Diets D12492) (HFD), low fat diet (Cargill Agri Purina Inc., Rodent Chow) (LFD) and autoclaved tap water were provided *ad libitum*, while the animals were housed at 23 °C, 55 ± 10 % humidity, in a 12 h light/dark cycle. All 120 mice were separated into 12 different groups each receiving different treatments (Table 1).

Table 1. Study design and animal treatments, based in a high-fat (HFD) and low-fat diet (LFD)

Group	Feed type	Treatment
LFD	LFD	300 µl PBS (non-obese control)
HFD	HFD	300 µl PBS (obese control)
Orlistat	HFD	40mg/kg suspended in 300 µl PBS
CJB38 L	HFD	1 x 10^8 CFU/day of *L. sakei* L338 suspended in 300 µl PBS
CJB38 M	HFD	1 x 10^9 CFU/day of *L. sakei* L338 suspended in 300 µl PBS
CJB38 H	HFD	1 x 10^{10} CFU/day of *L. sakei* L338 suspended in 300 µl PBS
CJB46 L	HFD	1 x 10^8 CFU/day of *L. sakei* L446 suspended in 300 µl PBS
CJB46 M	HFD	1 x 10^9 CFU/day of *L. sakei* L446 suspended in 300 µl PBS
CJB46 H	HFD	1 x 10^{10} CFU/day of *L. sakei* L446 suspended in 300 µl PBS
CJLS03 L	HFD	1 x 10^8 CFU/day of *L. sakei* LS03 suspended in 300 µl PBS
CJLS03 M	HFD	1 x 10^9 CFU/day of *L. sakei* LS03 suspended in 300 µl PBS
CJLS03 H	HFD	1 x 10^{10} CFU/day of *L. sakei* LS03 suspended in 300 µl PBS

The experiment comprised one week of adaptation followed by six weeks of obesity induction using HFD while the LFD group was maintained on LFD feeding. After six weeks as obesity induction period, each group was treated with either the PBS suspended microbial culture,

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PBS suspended Orlistat as chemical control or only PBS as negative control, twice a day at the same time (10:00 and 17:00) for seven weeks. On the last day of the experiment, the mice were sacrificed by dislocation of the cervical vertebrata. The organs, i.e., liver, femoral muscle, brown adipose tissue, epididymal adipose tissue, subcutaneous adipose tissue and mesenteric adipose tissue were collected, weighed, and kept at -80 ℃. Serum Triglyceride (TG), glucose (GLU), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), aspartate transaminase (AST) were measured using an automated biochemical analyser BS-200 (Mindray, China) in Pohang Technopark, Pohang (South Korea).

L. sakei strains CJB38, CJB46 and CJLS03 were grown daily in MRS broth (Difco Laboratories INC., Franklin Lakes, NJ, USA) for feeding during the seven weeks period of intervention. Strains were grown for 8 hours to reach their late log phase and were collected by centrifugation (3546 *g*, 5 min, 5 ℃) (Centrifuge: Hanil Science Industry, Korea) and washed two times with PBS. Each strain was prepared in an approximate number of 1 X 10^{10} CFU/ml using a mathematical equation derived from a pre-optimised standard curve (data not shown) using optical density by SPECTROstar Nano (BMG Labtech, Durham, USA). A stock suspension of 1 X 10^{10} CFU/ml was prepared of each strain, then diluted ten-fold to 1 X 10^{9} and 1 X 10^{8} CFU/ml, respectively, and finally suspended in 300 µl of PBS to be administered to each mouse by oral gavage.

Experimental determinants were statistically calculated using ANOVA and Dunnett's multiple comparison test to distinguish the level of significance based on probability of 0.05 (*), 0.01 (**) and 0.001 (***).

RESULTS

Three different doses (10^{8}-10^{10}) of the three *L. sakei* strains (CJB38, CJB46 and CJLS03) were orally administered to high fat diet induced obese C57BL/6 mice for 7 weeks, and body weight and food consumption were measured daily. During the test period, three strains were found to exhibit weight loss compared to the HFD group (Fig. 1 b, c, d). LFD, Orlistat, all of the CJB46 group, and medium and high dose of the CJLS03 groups showed significantly lower weight

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increase compared to the HFD group. The weight loss was not dependent of the dose of CJB38 or CJB46 while CJLS03 showed a dose-dependent weight reduction effect and CJLS03 H showed the highest efficacy of all groups (Fig. 1 e). The onset time of weight loss showed significance compared to HFD at days 4, 21, 21 and 7 for the Orlistat, CJB38, CJB46 and CJLS03 groups, respectively (data not shown). The daily dietary intake was significantly higher in the LFD, Orlistat and CJLS03 M groups compared to the HFD group (Fig. 1 f).

Serum biochemical analysis showed an overall increase in the lipid profile (TC, TG, HDL, LDL), liver (AST) and the glucose level of the HFD group compared to the LFD group, demonstrating that a high fat diet intake may impact various biomarkers associated with pathophysiological symptoms of obesity (Fig. 2). Compared to the HFD group, the serum TG level decreased in all test groups (Fig. 2 a) while the LDL level was significantly reduced in all test groups except CJB46 H (Fig. 2 e). Significant reduction of TC was only detected in LFD, Orlistat and in the groups treated with higher doses (M and H) of *L. sakei* CJB38 H, CJB46 M, CJB46 H, CJLS03 M and CJLS03 H (Fig 2 c). In particular, the CJLS03 group, shown to be superior regarding weight gain inhibition, appears to be effective in a dose dependent manner (Fig. 2 a, b, c). HDL levels were not significantly different from the HFD group in all the test groups, however, all *L. sakei* treated groups except CJB46 L, CJLS03 M and CJLS03 H showed significant increase when the ratio of HDL to total cholesterol level was calculated (data not shown). Serum AST values (indicating liver function) were found to be approximately 1.7 times higher for the HFD compared to the LFD group (Fig. 2 f), while the Orlistat group showed no significant change in AST level compared to the HFD group. All three groups receiving the *L. sakei* strains showed noticeable decrease of AST levels with a dose-dependent reduction in the CJLS03 groups, which was significant in the CJLS03 H group when compared to the HFD group (Fig. 2 f). CJLS03 showed the highest overall effectivity and a dose dependent anti-obesity function; at the same time, it induced a dose-dependent improvement of serum obesity associated biomarkers and liver function.

Compared to HFD the LFD group showed significantly lower weights of epididymal, mesenteric, subcutaneous and brown adipose tissues while insignificant organ weight

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differences were measured in liver and femoral muscles (Fig 3). Every dose of all three strains of *L. sakei* and the Orlistat group resulted in significantly lower subcutaneous adipose tissue weight while only CJLS03 H showed significant reduction of visceral adipose tissue including epididymal and mesenteric adipose tissue, when compared to the HFD group (Fig. 3 a, b, c). CJLS03 M treatment significantly reduced epididymal adipose tissue weight when compared to the HFD group (Fig 3 a). These results suggest that the three different *L. sakei* strains inhibited the accumulation of subcutaneous adipose tissue but that the CJLS03 group responded by dose dependent reduction of visceral adipose tissues including the epididymal and mesenteric adipose tissues (Fig. 3a, b). Orlistat and *L. sakei* treatment did not result in significant weight differences regarding brown adipose tissue, liver and femoral muscle (Fig 3 d, e, f).

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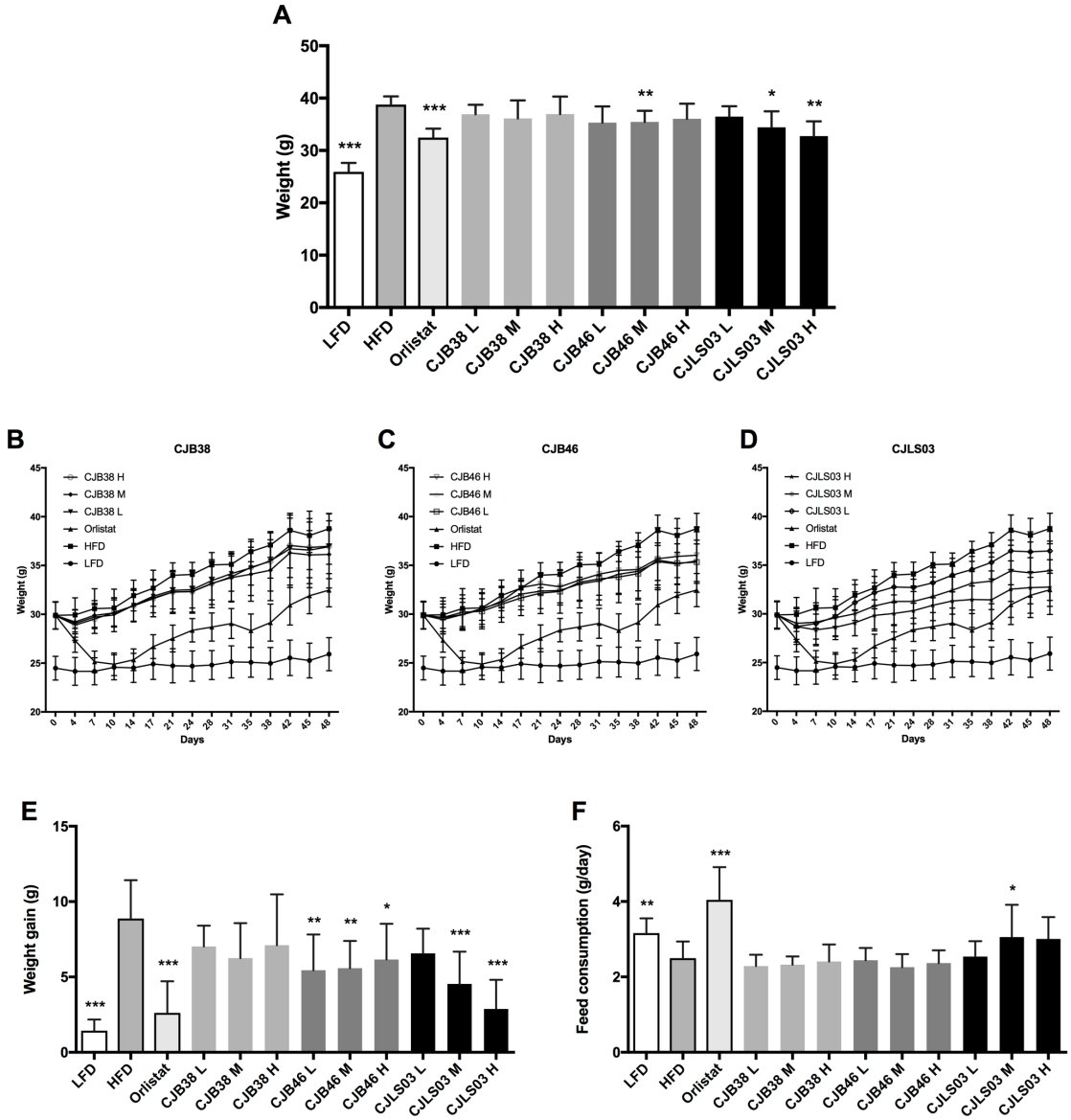

Figure 1 (A) Body weight after 48 days, (B, C, D) and increase over the 48-day period; (E) body weight gain after 48 days, and (F) daily feed consumption of each group. Asterisks denotes level of significant compared to HFD as *: p<0.05, **: p<0.01 and ***: p<0.001.

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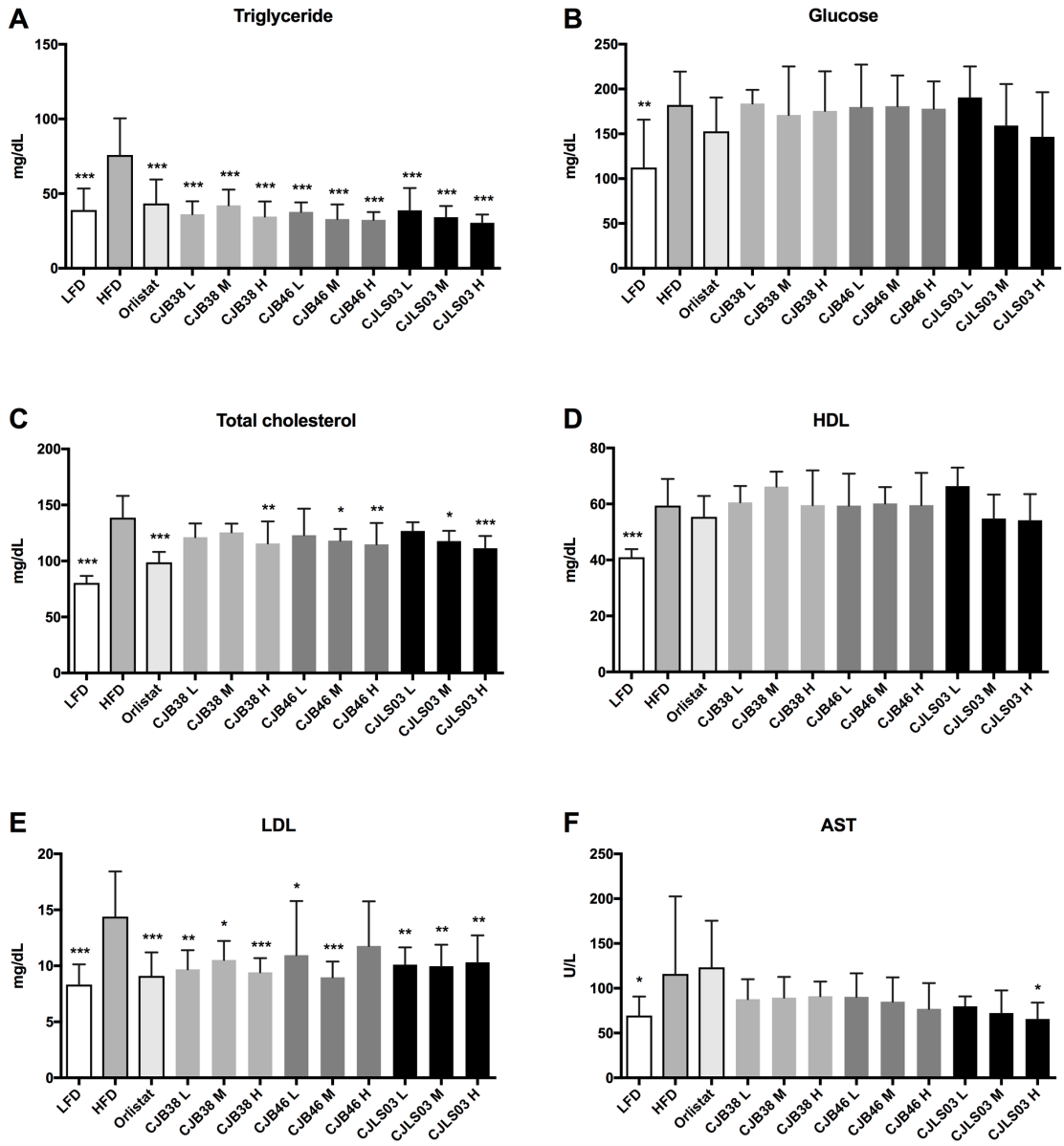

Figure 2 Serum biomarkers of each experimental group showing (A) triglyceride, (B) glucose, (C) total cholesterol, (D) high density lipoprotein (HDL), (E) low density lipoprotein (LDL) and (F) aspartate transaminase (AST). Asterisks denote the level of significance compared to HFD as *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

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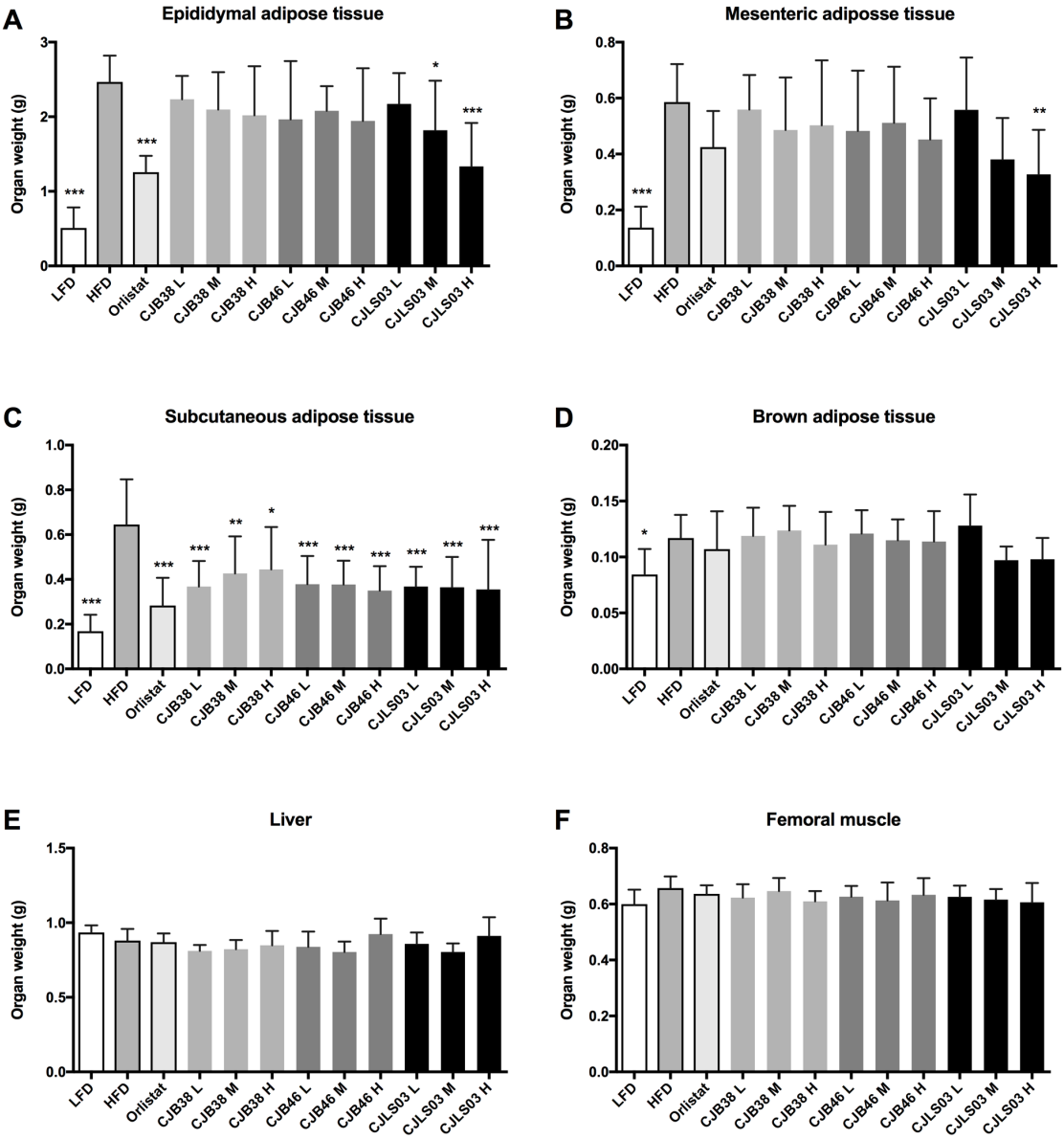

Figure 3 Organ weights of each experimental group showing (A) epididymal adipose tissue, (B) mesenteric adipose tissue, (C) subcutaneous adipose tissue (D) brown adipose tissue, (E) liver and (F) femoral muscle. Asterisks denote the level of significantce compared to HFD as *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

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DISCUSSION

The anti-obesity influence of administered probiotics is a heavily debated issue, yet, an indisputable fact is that host gut microbiota is exercising a leverage over energy efficiency and adipose tissue accumulation (*Kobyliak et al., 2017; Greiner and Bäckhed, 2011; Delzenne et al., 2011*); at the same time, probiotics have been reported to impact on host microbiota in a positive way.

Probiotic administration increasingly enjoys consideration as a promising approach for beneficially modulating the host microbiota (*Jia et al., 2008; Steer et al., 2000*). Numerous reports confirmed the beneficial effects of specific probiotic strains against diarrhoea and inflammatory bowel diseases (*Ahmadi, Alizadeh-Navaei & Rezai 2015; Gionchetti et al., 2000; Ouwehand, Salminen & Isolauri, 2002*). Recently, anti-obesity effects of probiotics were also reported and confirmed in clinical trials and animal models (*Kim et al., 2016; Alard et al,. 2016; Wang et al., 2015; Ji et al., 2012; Kadooka et al., 2010*). *Kadooka et al.* (2010) investigated the anti-obesity effect of the probiotic *L. gasseri* SBT2055 by conducting a double-blind, randomised, placebo-controlled intervention trial with 87 overweight and obese subjects for 12 weeks. The data confirmed that the abdominal visceral and subcutaneous fat area, weight, BMI, as well as waist and hip measures were significantly reduced in the group consuming the probiotic. In another study (*Woodard et al., 2009)* 44 morbid obese patients were operated for weight loss by surgery (gastric bypass surgery) and were randomly divided in a probiotic administered group and a control group. A significantly higher weight loss was recorded in the group receiving the probiotic. *Park et al. (2013)* reported a significant weight reduction of a C57BL/6 mice model after administering *L. curvatus* HY7601 and *L. plantarum* KY1032, however, faecal microbiota modulation of major groups such as *Firmicutes* and *Bacteroidetes* was not monitored.

One of the major hurdles for an accurate clinical trial is to understand the effective dose of a probiotic at a strain specific level. Selecting the correct dose of a probiotic for a specific

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purpose such as the alleviating of diarrhoea was suggested in various studies, yet, there is a general lack of scientific proof of a concept to define the functional dose of a probiotic (*Kollaritsch et al., 1993; Kollaritsch et al., 1989; Islam, 2016*). *Chen et al. (2015)* used 5 different tenfold doses of *L. acidophilus* in a colitis induced animal model and reported 10^6 CFU/10g of the animal weight as the most effective application level for modulating the bacterial profile in the distal colon.

In our study we have monitored the dose dependent anti-obesity effects of three different strains of *L. sakei* and found only one strain, CJLS03, to show a dose dependent anti-obesity effect while the anti-obesity impact of the other two strains was found to be dose independent. Adipose tissues were reduced relative to weight gain and triglyceride and total serum cholesterol showed the most significant reduction in the *L. sakei* treated groups compared to the HFD control group.

CONCLUSIONS

This *in vivo* investigation showed that beneficial effects of putative probiotics are both strain specific and dose related. For only one (CJLS03) out of three *L. sakei* strains an anti-obesity effect could be detected, which, at the same time, was found to be dose dependent. The highest of three doses (1 x 10^{10} CFU/day) of CJLS03 gave the most favourable (significant) biomarker related effects with regard to cholesterol and triglyceride reduction, when compared to the HFD control.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

YJ, SP, WHH have received research grants, via Handong Global University, from CJ CheilJedang

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Corp., Republic of Korea.

YC, DJ, BK are employed by CJ CheilJedang Corp., Republic of Korea.

Author Contributions

- Yosep Ji, Young Mee Chung and Soyoung Park were equally involved in designing and conducting the experiments and are jointly first co-authors.
- Yosep Ji, Young Mee Chung and Soyoung Park analysed the data, prepared the figures and tables and drafted the first version of the paper.
- Dahye Jeong, Bongjoon Kim, Wilhelm H. Holzapfel and Yosep Ji conceived the experiments, contributed reagents/materials/analysis tools, and reviewed drafts of the paper together with Soyoung Park.

Animal Ethics

The animal study was approved by the Ethical Committee of KPC Ltd. in Korea (P150067) in full compliance with ethical standards as specified by Korean law.

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