

# High-fat diet-induced oxidative stress links increased colonisation of *Lactobacillus sakei* with its anti-inflammatory properties in obesity

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## Research

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# Abstract

Background: Obesity is one of the major public health problems related to various chronic health conditions, with steadily increasing prevalence worldwide. *Lactobacillus* provides various benefits to the host body; however, its role in obesity is unknown.

Results: In this study, we found higher colonisation of *Lactobacillus sakei* species in obesity group, which in turn was related to increased reactive oxygen species (ROS) levels induced by higher fat intake. We isolated *L. sakei* ob4.1 strain from the faeces of one subject with obesity and compared its genetic and molecular features with those of *L. sakei* DSM 20017 strain. *L. sakei* ob4.1 showed higher catalase activity, which was regulated by oxidative stress at gene transcriptional levels. *L. sakei* ob4.1 maintained colon epithelial cell-adhesion ability under ROS stimulation, and live bacteria could decrease colon epithelial inflammation in a dose-dependent manner. Establishing a mouse model revealed high-fat diet-induced colon ROS to be associated with increased colonisation of *L. sakei* ob4.1 through catalase activity. Four-week supplementation with this strain could reduce colon inflammation effectively, though not so for body weight or ROS levels in high fat-fed mice.

Conclusion: We, therefore, concluded that changes in host gut-oxidative stress levels could link high fat-induced obesity to increased colonisation of *L. sakei* ob4.1, and this strain could be potent as anti-inflammatory probiotic in obese population with gut inflammation. Keywords *Lactobacillus sakei*; Obesity; Gut microbiome; Reactive oxygen stress; Inflammation; High fat diet

## Introduction

Obesity is a multifactorial disease that involves both genetic and environmental factors[1, 2], hence necessitating the identification of its risk and preventive factors in order to reduce the possibility of obesity and obesity-related complications.

Probiotics are live microorganisms with benefits on host health when administered in adequate amounts[3]. The genus *Lactobacillus* has been reported to be the most commonly used probiotics. Some strains of *Lactobacillus* have shown beneficial effect on obesity and obesity-related complications[4–6], in clinical trial, whereas higher colonisation of *Lactobacillus*[7], particularly *Lactobacillus sakei* [8], has also been reported in obese population. Hence, the role of *Lactobacillus* in obesity is controversial and varies across strains.

The relationship between gut microbiota and obesity has been widely studied till date[9, 10]. Although the precise role of gut microbiota in obesity is still unknown, a dietary factor, especially high fat consumption, is considered to play an important role in linking obesity with gut microbiota. High-fat diet is known to

increase the levels of oxidative stress in colon[11, 12] and affect the composition of colonic microbiota[13, 14]. Abdominal fat tissue is also known to directly increase reactive oxygen species (ROS) levels[15, 16] *Lactobacillus* has been reported to show high diversity in resistance to oxidative stress across species[17, 18] and strains[19], based on catalase activity. Hence, it may be hypothesised that colonisation of different *Lactobacillus* strains can be determined by the oxidative stress levels in colon, induced by high-fat diet or obesity. However, the role of obesity or high-fat diet in colonisation of *Lactobacillus* has never been studied till date. In this study, we identified and isolated the *Lactobacillus* species related to obesity and high-fat diet, and further investigated the characteristics and probiotic potential of this strain related to colonic oxidative stress in obesity.

## Materials And Methods

**Study participants.** Participants were recruited from the individuals that visited the hospital (Department of Family Medicine of Severance Hospital and CHAUM) for either regular health check-up or reducing body weight. Obese subjects were defined by body mass index (BMI)  $> 25 \text{ kg/m}^2$ , and control subjects were healthy participants over 19 years of age, with BMI between 19 and 23  $\text{kg/m}^2$ . We excluded the participants with history of any type of cancer, inflammatory bowel diseases, or abdominal surgery. Subjects with history of taking probiotics or antibiotics, within 4 weeks prior to participation, were also excluded. Overall, 64 healthy control subjects and 88 obese subjects were included in the final analysis.

Information about Questionnaire, assessment of nutrient intake and measurements can be found in the supplementary methods details.

**Stool sample collection.** Participants were provided sterile plain tubes, without any chemical additive, for stool collection. Approximately 2 g of fresh stool samples were collected and transported as soon as possible to the laboratory; 100 mg of fresh stool was used for *Lactobacillus* culture study and the rest was stored at  $-80 \text{ }^\circ\text{C}$  for subsequent analysis.

**Human faecal ROS measurement.** Approximately 100 mg of faeces were homogenised in phosphate buffered solution (PBS), and centrifuged at 3000 rpm at  $4 \text{ }^\circ\text{C}$  for 20 min. The supernatant was used for analysis. Human faecal ROS level was measured using a commercial enzyme-linked immunosorbent assay kit (Human ROS ELISA kit, MyBioSource Inc.), according to the manufacturer's instruction.

## 16S rRNA quantitative PCR analysis of total *Lactobacillus*

Genomic DNA was extracted from the faeces using a QIAmp DNA Stool Mini Kit (Qiagen), according to the manufacturer's protocol. Relative abundance of *Lactobacillus* was analysed by quantitative real-time PCR, as described by Yoon et al[20]. Briefly, 2  $\mu\text{L}$  of 10-fold-diluted genomic DNA, extracted from the faeces, were taken as the template; SYBR Green PCR master mix (Applied Biosystems) and appropriate primer sets designed to amplify the 16S region [21] (Table S3) were used for performing quantitative real-

time PCR. Transcript levels of target genes were normalised to gene expression levels of the housekeeping gene *gapdh*.

PCR analysis of *Lactobacillus* species. Genomic DNA was extracted from the faeces using QIAmp DNA Stool Mini Kit (Qiagen), according to the manufacturer's protocol. A reaction mixture consisted of 100 ng of bacterial DNA from the faeces, PCR Supermix High Fidelity (Invitrogen), and appropriate primer sets designed to amplify 16S–23S ribosomal RNA intergenic spacer region[22–25]. Amplified PCR products were detected by agarose gel electrophoresis using a 1.5% agarose gel, with ethidium bromide staining and UV transillumination.

Isolation of *L. sakei* strains from faeces. For the isolation of *L. sakei* strains in faeces, 100 mg of fresh stool were suspended in 900  $\mu$ L of PBS and homogenised. The homogenised sample solutions were serially diluted ( $10^{-2}$  –  $10^{-6}$ ), 50  $\mu$ L of each dilution plated in duplicate on the deMan-Rogosa-Sharpe (MRS) medium (Difco), and finally incubated anaerobically at 37 °C for 48–72 h. For excluding non-lactic acid-producing bacteria, each colony with a different morphology was isolated and plated on the MRS medium with 1%  $\text{CaCO}_3$  and incubated anaerobically at 37 °C for 48 h. Bacteria that produced clear zones around colonies were selected. The presence of *L. sakei*-specific *kata* gene was determined by PCR amplification, followed by the isolation of *L. sakei*, using the method previously described[26]. Thereafter, Sanger sequencing of 16S rRNA gene was performed for the confirmation of *L. sakei*. Genomic DNA was isolated from each colony using G-Spin Genomic DNA Extraction Kit (iNtRON Biotechnology Inc.), according to the manufacturer's instruction. The isolated genomic DNA was used in a PCR reaction to amplify the 16 s rRNA gene using primers 27F-1492R. After purification, using QIAquick PCR purification kit (Qiagen), PCR products were sequenced by Macrogen Inc. The nucleotide sequences were analysed for sequence similarity, using BLAST (<http://www.ncbi.nlm.nih.gov/blast>), and sequences with  $\geq 98\%$  match with those in database were considered to be from the same species. Results showed that only one strain was found from the faeces of obese participant. Information about whole genome sequencing and comparative genomic analysis are provided in the supplementary methods details.

Survival rate under hydrogen peroxide challenge test. Exponential-phase bacterial cells were centrifuged ( $6000 \times g$ , 15 min), re-suspended in MRS medium containing 0, 5, 10, 15, and 30 mM  $\text{H}_2\text{O}_2$ , and incubated thereafter at 37 °C. After 1 h,  $\text{H}_2\text{O}_2$  was eliminated using bovine liver catalase (10 U/mL, Sigma), and viable cells were counted by plating appropriate dilutions on MRS medium.

Growth conditions of *L. sakei* strains. Overnight-grown cultures of *L. sakei* ob4.1 or DSM 20017 were diluted in MRS medium (1:1000) and incubated at 37 °C either aerobically using a shaking incubator (200 rpm), or anaerobically in an anaerobic jar with GasPak (BD). CFUs were determined by measuring optical density at 600 nm every 4 h for 3 days.

Detection of catalase activity in *Lactobacillus* strains. Catalase activity was analysed as described previously[27]. Briefly, *Lactobacillus* strains were incubated in MRS medium with 30  $\mu$ M haematin (Sigma), either aerobically using shaking incubator (200 rpm), or anaerobically in anaerobic jar with

GasPak (BD). The exponential growth-phase bacterial cells ( $OD_{600} = 0.5$ ) were harvested and re-suspended in PBS at  $10^8$  CFU/mL. The re-suspended cells were mixed with 0.8 mM hydrogen peroxide. An aliquot from there was mixed with dichromate in acetic acid, and the samples were boiled and centrifuged to remove cells. Absorbance was measured at 570 nm. Catalase activity was expressed in terms of  $\mu\text{M H}_2\text{O}_2$  degraded per minute per  $10^8$  CFU.

Expression of catalase genes in *L. sakei* strains. RNA isolation and cDNA synthesis were performed as described previously [28]. TRIzol® Max™ Bacterial RNA Isolation Kit (Invitrogen) was used for RNA isolation, according to the manufacturer's instruction. Sequences encoding catalase of *L. sakei* ob4.1 were used as templates for primer design (Table S3). Relative expression of *katA* gene was calculated using the comparative threshold ( $\Delta\Delta\text{Ct}$ ) method. *Gapdh* was used as a reference gene.

Caco-2 cell culture. Caco-2 cells (ATCC) were grown on Minimal Essential Medium (MEM) (Gibco) containing 10% foetal bovine serum, 1% glutaMAX (Gibco), 1% MEM non-essential amino acids (Gibco), and 1% sodium pyruvate at 37 °C and 5%  $\text{CO}_2$ . After confluency, the cells were seeded into 6- or 96- well plates, for individual experiments, at a density of  $1 \times 10^5$  cells/well and cultured for 21 days (on an average) with medium change every alternate day, until fully differentiated.

Preparation of bacterial suspension. *Lactobacillus* strains were grown anaerobically in MRS medium for 48 h at 37 °C, and bacterial cultures were pelleted down and resuspended in MEM medium, with a final concentration of  $10^8$  CFU/mL. Heat-killed bacteria were prepared by heating bacteria at 80 °C for 15 min at a concentration of  $10^{10}$  CFU/L in MRS medium. Samples, before and after the killing procedure, were diluted in PBS, plated on MRS agar, and incubated at 37 °C for 72 h.

Palmitate treatment. Differentiated Caco-2 cells were treated with palmitate (Sigma Aldrich) (5 mM) or fatty acid-free bovine serum albumin (Sigma Aldrich), and incubated at 37 °C and 5%  $\text{CO}_2$  for 6 h. Suspensions of *Lactobacillus* strains containing  $10^8$  CFU/mL cells were added to each well and incubated for another 4 h. Caco-2 cells were washed twice with DPBS, and cell pellets were resuspended in 1 mL of TRIzol reagent (Molecular Research Centre) for subsequent RNA extraction. Detailed information about real-time PCR analysis can be found in the supplementary methods details. Information about adhesion assay and ROS measurement are provided in the supplementary methods details.

Mouse experiments. The Institutional Animal Care and Use Committee at the CHA University approved the animal experiments used in this study. Specific pathogen-free male C57BL/6J mice, aged 6 weeks, were fed either 10% control diet (D12450B, Research Diets, Inc.) or a 60% fat diet (D12492, Research Diets, Inc.) until the end of the experiment. Two weeks after starting the diet,  $1 \times 10^9$  CFU/100  $\mu\text{L}$  of *L. sakei* ob4.1 or DSM 20017 were administered by oral gavage, once, and colonisation of bacteria in mouse faeces was determined by homogenising the latter in 1 mL of sterile PBS, serially diluting the samples, and eventually plating them on MRS medium plates. For investigating the probiotic effect of strains, mice were supplied with  $1 \times 10^9$  CFU/100  $\mu\text{L}$  mixture of indicated *L. sakei* strains or mock, which was orally administered

every day for 4 weeks. Mice were subsequently euthanised, and samples were collected 24 h after the final infection (12 weeks old). Information about bacterial culture, colon tissue ROS measurement and real-time PCR analysis are provided in the supplementary methods details.

Statistical analysis. Detailed information about statistical analysis can be found in the supplementary methods details

## Results

### Lactobacillus sakei was increased in obesity group.

In this study, we enrolled 88 adults with obesity and 64 healthy control subjects without obesity. No significant difference was found between the obesity and control groups, with respect to age, gender, and social habits, except for diet.; higher intake of daily calories, calorie-adjusted fat, and calorie-adjusted saturated fat was found in the obesity group (Table S1). In order to compare the abundance and composition of *Lactobacillus* species between the two groups, we quantified the relative abundance of *Lactobacillus* in their faeces, using real-time polymerase chain reaction (PCR), and found no difference between the two groups (Fig. 1a). Considering the high diversity across *Lactobacillus* species to obscure the relationship between obesity and *Lactobacillus*, we performed species-specific PCR analysis next. Obesity group was more often colonised with *L. sakei* than the control group (Fig. 1b). There was no significant difference in the prevalence of other *Lactobacillus* species between the two groups (Fig. 1b).

### Relationship between fat consumption and *L. sakei* abundance

Since higher fat consumption was seen in the obesity group than in the control group, we analysed the relationship between fat consumption and *L. sakei* abundance. High fat consumption is one of the main reasons of obesity, and affects the composition of gut microbiota, independent of the development of obesity[29, 30]. We categorised all participants into four groups according to the quartiles of fat consumption. Notably, participants in the highest fat-consumption group were approximately 3.1 times (odds ratio [OR]: 3.21, confidence interval [CI]: 2.03–4.57) more likely to have *L. sakei* strain in their faeces than those in the lowest fat-intake group, after adjusting for age, gender, and body mass index (BMI) (Fig. 1c). Saturated fatty acids increase the reactive oxygen species (ROS) levels in gut[11, 12] and *L. sakei* contains haem-dependent catalase, despite most *Lactobacillus* species being catalase negative[26, 31]. Hence, we hypothesised that the catalase activity of *L. sakei* might be a mechanistic underpinning of the relationship between high-fat diet and *L. sakei*. To investigate this hypothesis, we measured ROS levels in the faeces of each participant. We could find a trend of increased ROS levels in the faeces from obesity group compared to that from the control group ( $p = 0.06$ ) (Fig. 1d). When categorising the participants according to fat intake, ROS levels in faeces were found to increase significantly with increase of fat intake, after adjusting for BMI (Fig. 1e). It, therefore, suggested that higher levels of ROS might arise from fat intake, rather than from obesity itself.

### Lactobacillus sakei strain was isolated from the faeces of obese subjects

To further investigate the characteristics of *L. sakei*, related to high-fat diet-induced ROS, we isolated *L. sakei* strain from the faeces of obese subjects. Fresh faeces from 15 subjects in the obesity group were used for this purpose. *Lactobacillus*-specific culture, for confirming the presence of *L. sakei*-specific *katA* gene, and 16S rRNA gene sequencing, were used for isolation. Only one strain of *L. sakei* was isolated from one subject, and we named it *L. sakei* ob4.1. Severe obesity (BMI 32.15 kg/m<sup>2</sup>) and high fat consumption (37.28 g/1000 kcal/day) were found in the host of *L. sakei* ob4.1 (Table S2).

### ***L. sakei* ob4.1 showed higher resistance to oxidative stress than *L. sakei* DSM 20017**

Since our data suggested high fat intake to increase ROS levels in the faeces, we aimed to explore the resistance of *L. sakei* ob4.1 against oxidative stress.

*L. sakei* is known to show high diversity, regarding catalase activity, across the strains[19]. For comparison, we chose *L. sakei* DSM 20017 as a reference strain, (isolated from rice wine), whose whole genome information was available[32]. For negative control, we used *L. rhamnosus* GG (ATCC53103), which contained no catalase gene[33]. Survival of each strain was assessed under oxidative stress, generated by either H<sub>2</sub>O<sub>2</sub> or aeration. The short-term survival ratio of *L. sakei* ob4.1 was significantly higher than that of *L. sakei* DSM 20017 in presence of each concentration of H<sub>2</sub>O<sub>2</sub> (5, 10, 15, and 30 mM) (Fig. 2a, b). Long-term survival of *Lactobacillus* strains, under aerobic conditions, was evaluated next. After 24 h of aerobic growth, survival of *L. sakei* ob4.1 was approximately 100 times higher than that of *L. sakei* DSM 20017 (Fig. 2c). Next, we measured the adhesion ability of *L. sakei* strains, with or without oxidative stress. Compared to *L. rhamnosus* GG, which is known to have great adhesion ability, both the *L. sakei* strains showed similar adhesion properties without H<sub>2</sub>O<sub>2</sub>. However, after H<sub>2</sub>O<sub>2</sub> treatment, *L. sakei* ob4.1 was the only strain that could maintain adhesion ability (Fig. 2d).

### **Functional and comparative genomic analysis of *L. sakei* ob4.1**

In order to understand the genetic basis of the higher resistance of *L. sakei* ob4.1 to oxidative stress, we sequenced the genome of *L. sakei* ob4.1 and compared it with that of *L. sakei* DSM 20017. *L. sakei* ob4.1 was found to have larger genome (2.03 Mbp) than *L. sakei* DSM 20017 (1.91 Mbp) (Supplementary Fig. 1a). Maximal Unique Matches algorithm[34] was used to align the two strains, and high degree of dissimilarity was observed between them (Supplementary Fig. 1b). Next, we performed genome-based clustering analysis using *L. sakei* ob4.1, *L. sakei* DSM 20017, and 15 other representative *L. sakei* strains (Supplementary data 1). *L. sakei* ob4.1 and DSM 20017 were relatively distant from each other, with average nucleotide identity (ANI) of 98.73% (Supplementary Fig. 2). It indicated that the two strains were from different phylogenetic lineages within the *L. sakei* species. We next investigated the strain-specific genes. A total of 207 genes were present in *L. sakei* ob4.1, though not in *L. sakei* DSM 20017 (Supplementary Fig. 1c). We next investigated the genes involved in oxidative stress, in *L. sakei* strains, from among those that associated with higher catalase activity of *L. sakei* ob4.1. However, we could not find any difference in the presence or absence of genes involved in oxidative stress, across the *L. sakei*

strains (Supplementary data 2). Each *L. sakei* strain contained one catalase gene (*KatA*), and 98.5% identity was found across amino acid sequences of the catalase (Supplementary Fig. 1d).

#### ***L. sakei* 4.1 had higher catalase expression and activity under oxidative stress**

We could not identify genetic factors that explained the higher resistance of *L. sakei* ob4.1 to oxidative stress. However, considering that catalase activity is regulated by oxidative stress at gene transcription and protein synthesis levels in various *Lactobacillus* species[31, 35], we investigated the effect of oxidative stress on *katA* mRNA level and catalase enzymatic activity in *L. sakei* strains. Increased *katA* mRNA expression was detected in both strains, grown in aerobic conditions rather than in anaerobic conditions, as well as in strains treated with hydrogen peroxide. However, the increase was significantly higher in *L. sakei* ob4.1 (Fig. 3a). Next, we measured the catalase activities of *Lactobacillus* strains. Significantly higher increase of catalase activity was found in *L. sakei* ob4.1 grown in aerobic conditions (Fig. 3b). These results collectively suggested that resistance of *L. sakei* ob4.1 to oxidative stress could be regulated by the extent of oxidative stress at the transcriptional level, rather than at the genetic levels.

#### ***L. sakei* ob4.1 strain reduced palmitate-induced inflammation in colon epithelium**

In order to identify the roles of *L. sakei* ob4.1 strain in host colon epithelium, we focused on the anti-inflammatory properties of *L. sakei* ob4.1 in colon epithelial cells. The characteristics of *L. sakei* are diverse across the strains, with only a few strains showing anti-inflammatory properties[36–38]. In comparative genomic analysis, *L. sakei* ob4.1 had the highest similarity with *L. sakei* proBio65 strain (Supplementary Fig. 2), which was isolated from fermented vegetable (kimchi) and is used as a probiotic strain with anti-inflammatory effects[37]. First, we stimulated Caco-2 cells with a saturated fatty acid (palmitate). Palmitate stimulation significantly increased ROS levels in colon epithelium (Fig. 4a), and gene expression of proinflammatory cytokines like IL-6 (Fig. 4b) and TNF-alpha (Fig. 4c). When treated with live cells, *L. sakei* ob4.1 strain decreased gene expression of pro-inflammatory cytokines significantly, comparable to *L. rhamnosus* GG (Fig. 4b, c). ROS levels were sustained high after treatment with *L. sakei* strain (Fig. 4a). When treated with heat-killed cells, *L. sakei* ob4.1 and *L. sakei* DSM 20017 showed no significant change while *L. rhamnosus* GG maintained the anti-inflammatory functions (Supplementary Fig. 3a, b). When Caco-2 cells were treated with different doses of *Lactobacillus* strains, we found anti-inflammatory properties to be dose-dependent, both in *L. sakei* ob4.1 and DSM 20017 (Supplementary Fig. 3c,d). These results suggested that maintenance of cell viability is essential for anti-inflammatory properties of *L. sakei* strains, and that the latter could be mediated by a yet unknown mechanism, not by reduction of ROS in colon epithelium.

#### ***L. sakei* ob4.1 showed high colonisation and reduced colon inflammation in high-fat diet-induced obese mice**

To investigate the interaction between *L. sakei* ob4.1 strain and host colon *in vivo*, mice maintained on a high-fat (60%) or low-fat (10%) diet for 2 weeks were inoculated independently with  $1 \times 10^8$  CFU/mouse of spontaneous rifampin-resistant *L. sakei* strains. After 2 days of gavage, bacterial numbers in the colon

contents were counted. *L. sakei* ob4.1-inoculated mice on a high-fat diet shed *L. sakei* significantly in the colon contents compared to *L. sakei* DSM 20017-inoculated mice on a high-fat diet (Fig. 5a). Total number of *Lactobacillus* was not different between the strains, in mice maintained on low-fat diet (Fig. 5a). This result was consistent with the higher attachment ability of *L. sakei* ob4.1 in palmitate-treated Caco-2 cells. When we investigated whether *L. sakei* ob4.1 had anti-inflammatory properties in high-fat-induced obese mice, if administrated as a probiotic, the mice on high-fat diet exhibited significantly higher colon mucosal ROS levels (Fig. 5c). Furthermore, reduced colon length (Fig. 5d) and increased expression levels of inflammatory markers of colon mucosa (Fig. 4e, f) were found in the high-fat-fed mice. Following 4 weeks of treatment with *L. sakei* ob4.1 or *L. sakei* DSM 20017, body weight did not change in the mice, with either low-fat diet or high-fat diet (Fig. 5b). Neither of *L. sakei* ob4.1 or *L. sakei* DSM 20017 decreased ROS levels in colonic mucosa (Fig. 5c), although *L. sakei* ob4.1 significantly increased the colon length (Fig. 5d) and decreased inflammatory marker expression in high-fat-fed mice (Fig. 5e). Together, these results show that effects of *L. sakei* ob4.1 observed in our obese mouse model are similar to those in the palmitate-induced colon epithelial cells.

## Discussion

*L. sakei* belongs to the genus *Lactobacillus*, which is known to be present in raw meat and widely used as a starter for fermentation of sausage[39–41]. Recently, *L. sakei* has been found in human faeces[42, 43], and could be related to human diet, such as higher consumption of meat. Although most *Lactobacillus* strains are sensitive to oxidative stress, caused by hydrogen peroxide, *L. sakei* is one of the strains that are highly resistant to the same via catalase activity[26, 31]; there is, however, a high degree of intraspecies diversity regarding the response to oxidative stress[19]. In our study, we isolated an *L. sakei* strain (*L. sakei* ob4.1) from the faeces of humans with severe obesity, and the strain showed significantly higher resistance to oxidative stress relative to that in a reference strain *L. sakei* DSM 20017 isolated from rice wine.

Oxidative stress occurs as a result of an imbalance between reactive oxygen species and the defence system responsible for ROS elimination. Long-term high-fat diet induces oxidative stress in colon[11, 12] besides inducing bacterial dysbiosis by changing the redox status[13, 14]. Bacteria with higher resistance to oxidative stress (e.g. *E. coli*) have a higher chance to survive[14]. In our study, *L. sakei* ob 4.1, with a higher resistance to oxidative stress, showed superior survival rate than *L. sakei* DSM 20017 *in vitro*, and higher colonisation in the colon of high-fat diet-fed mice. These results collectively suggested higher catalase activity of *L. sakei* ob4.1 to enable the strain to survive better in the colon under high oxidative stress. We could not figure out the precise molecular basis that could explain the higher catalase activity of *L. sakei* ob4.1; however, increased gene transcriptional levels and catalase activity, under oxidative stress, suggested the possibility of it being regulated, at transcriptional and translational levels, by the level of oxidative stress.

Although many *Lactobacillus* strains have been widely used as probiotics, only a few studies have reported the probiotic properties of *L. sakei* strains[36–38]. In our current study, *L. sakei* ob4.1 reduced the

inflammatory cytokine levels both *in vitro* and *in vivo*. Anti-inflammatory effects were dose-dependent, and dead bacteria did not reduce the colon inflammation, although the effect was maintained in *L. rhamnosus* GG. This observation suggested live bacteria to be important for the probiotic effect of *L. sakei* strain ob4.1. High-fat diet is a risk factor for diseases related to colon inflammation (e.g. ulcerative colitis), as well as obesity[44, 45]. Mitochondrial dysfunction and increased oxidative stress, induced by high-fat diet, further contributed to bacterial dysbiosis, and supplementation by *Lactobacillus* strains was found to be effective in reducing colon inflammation in many studies[46]. However, attachment of *Lactobacillus* strains to colon mucosa was temporary, and persistence of colonisation of *Lactobacillus* strains in colon[47], especially in inflamed gut, was difficult to induce. In our study, *L. sakei* ob4.1 maintained adhesion ability under ROS stress *in vitro* and showed higher colonisation in high-fat-induced obese mice. Furthermore, 4 weeks of *L. sakei* ob4.1 supplementation significantly reduced the colon inflammation induced by high-fat diet. These results indicated the potential of this strain as a probiotic, aiming at improving colon inflammation in obese population.

The current study had several limitations. Cross-sectional relationship between *Lactobacillus* strains and obesity group could not find causality. We have compared *L. sakei* ob4.1 with a reference strain isolated from food, rather than with a strain isolated from control group. The underlying mechanism of high catalase activity and anti-inflammatory effects of *L. sakei* ob4.1 still remain unknown. In our study, *L. sakei* ob4.1 did not decrease colon ROS levels, induced by high-fat diet, thus suggesting anti-inflammatory properties of this strain to not be induced by decreasing ROS levels, rather via a different mechanism.

## Conclusions

In conclusion, higher catalase activity of *L. sakei* ob4.1 strain, isolated from subjects with obesity, was found to induce higher colonisation of this strain in colon, under high-fat-induced oxidative stress. Anti-inflammatory properties of this strain suggested its potential as a probiotic.

## List Of Abbreviations

Lactobacillus sakei, *L. sakei*; Lactobacillus DSM 20017, *L. DSM 20017*; Lactobacillus Rhamnosus GG, *L. rhamnosus* GG; Reactive oxygen species, ROS; Phosphate buffered solution, PBS; deMan-Rogosa-Sharpe, MRS; Minimal Essential Medium, MEM; Polymerase chain reaction, PCR; Odds ratio, OR; Body mass index, BMI

## Declarations

### Ethics approval and consent to participate

All subjects participated in the study voluntarily and provided written informed consent was obtained from each subject. The study complied with the Declaration of Helsinki and was approved by the

Institutional Review Board of Yonsei University College of Medicine and CHA Bundang Medical Centre.

## **Consent for publication**

Not applicable

## **Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files. The raw datasets are available from the corresponding author on reasonable request.

## **Competing interests**

The authors declare that they have no competing interests.

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## **Authors' contributions**

Conceptualization, J.-Y.L., K.K., D.-C.L. and S.S.Y.; Methodology, J.-Y.L. and S.S.Y.; Validation, E.B. and K.-M.L. ; Formal Analysis, J.-Y.L.; Investigation, J.-Y.L., E.B., H.Y.K. and K.-M.K.; Resources, D.-C.L. and S.S.Y.; Writing, J.-Y.L., S.S.Y and D.-C.L.; Supervision, S.S.Y and D.-C.L.; Funding Acquisition, J.-Y.L., S.S.Y and D.-C.L.

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## Figures

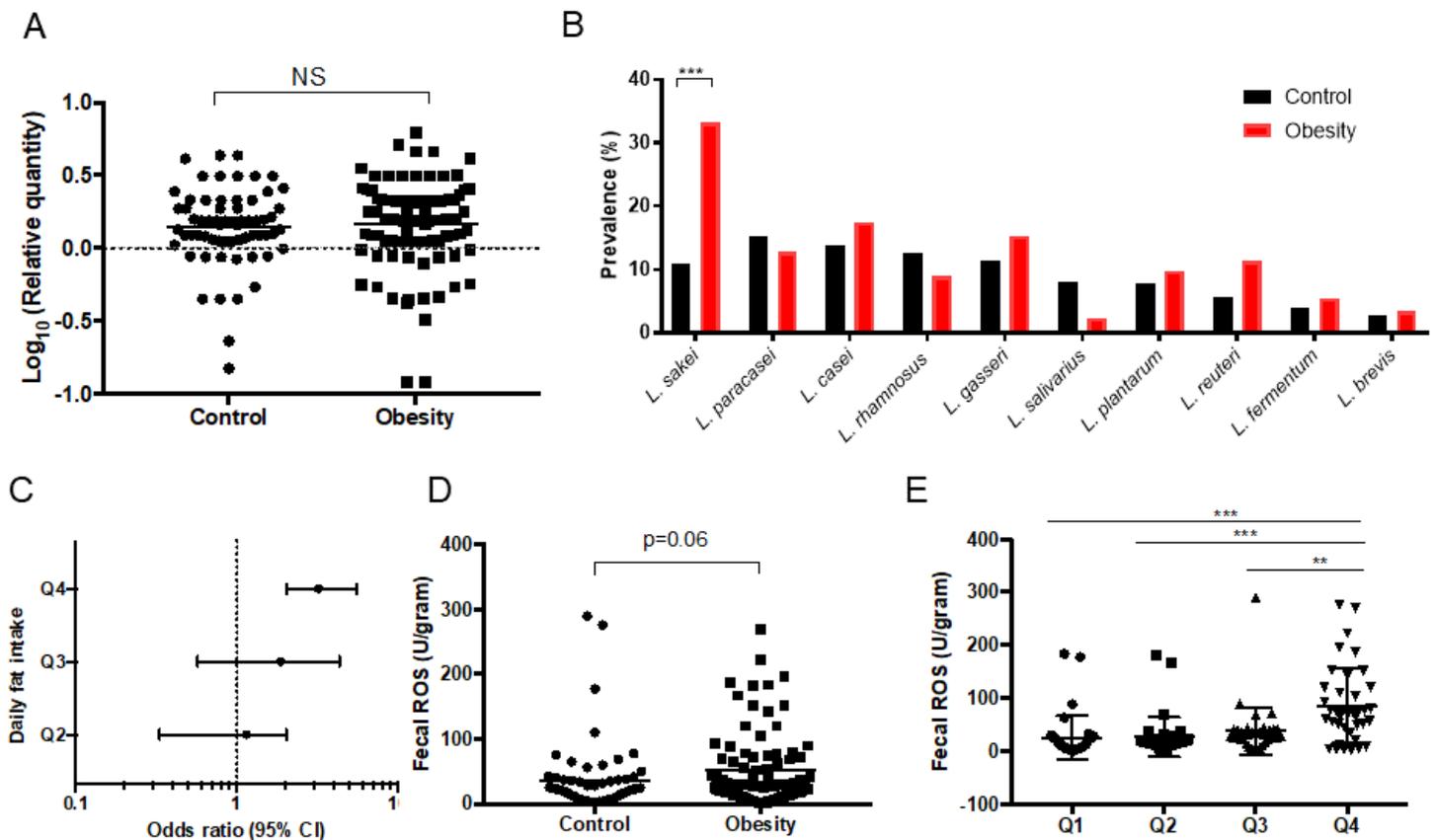


Figure 1.

## Figure 1

Association of high fat intake in obesity group with increased gut oxidative stress and *Lactobacillus sakei* colonisation. A total of 64 healthy control subjects (BMI between 19 and 23 kg/m<sup>2</sup>) and 88 obese subjects (BMI > 25 kg/m<sup>2</sup>) participated in the study. Faecal samples were collected from each participant, and all participants completed a questionnaire regarding food intake. (A) Relative abundance of *Lactobacillus* genus in faeces was determined by real-time PCR targeting the 16S rRNA gene. (B) The presence of *Lactobacillus* species in faeces was determined by species-specific PCR. (C) Odds ratio and

95% CI for the prevalence of positive culture of *L. sakei* species were calculated by multivariate logistic regression analysis after adjustment for age, gender, and BMI. Nutritional analysis was performed using CAN-PRO software 5.0. (D, E) Levels of faecal ROS were measured in the faeces by ELISA. Each symbol represents data from one individual subject (A, D, E), each line represents odds ratio with 95% confidence interval (B). \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; NS,  $P > 0.05$ .

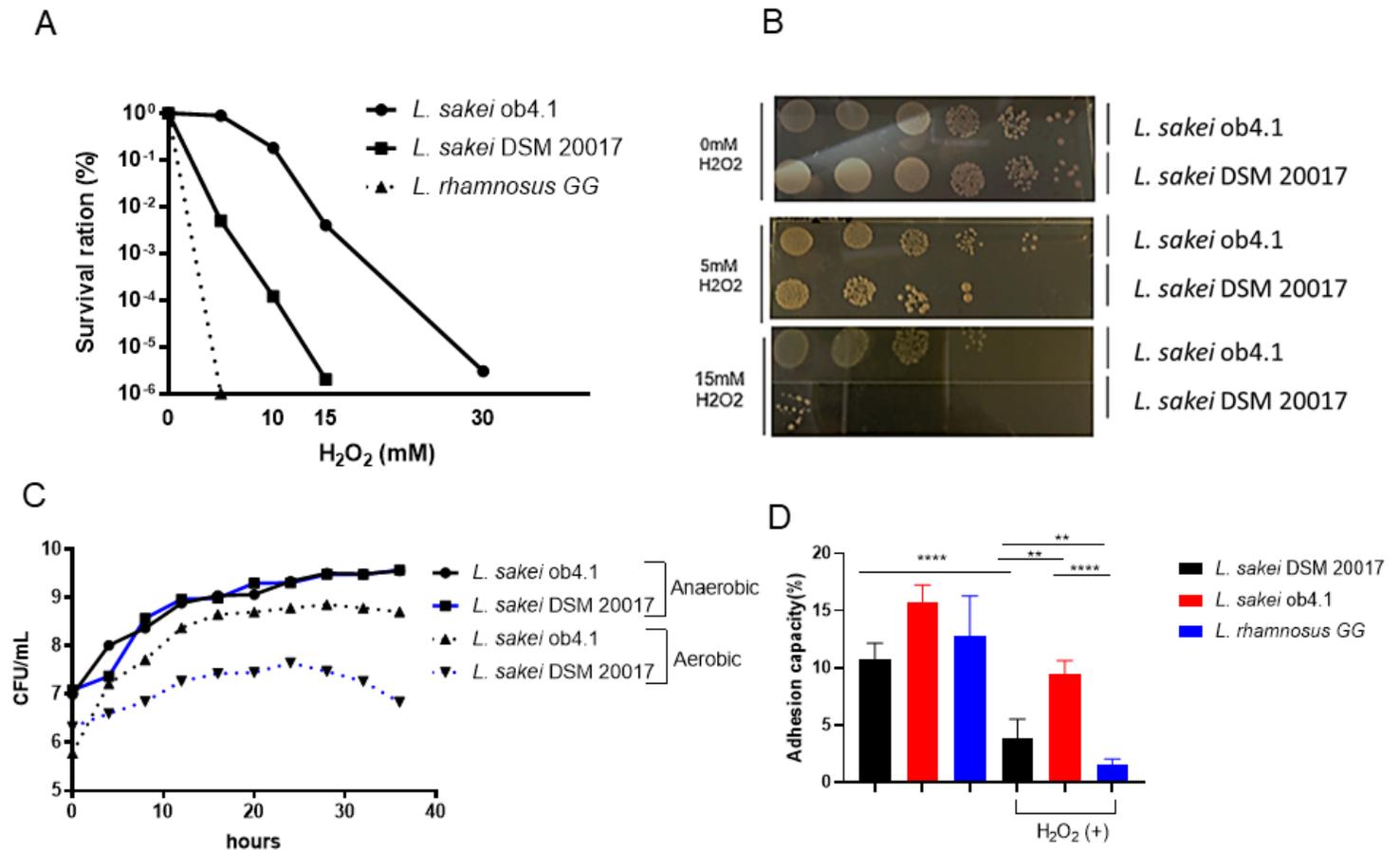


Figure 2.

## Figure 2

Lactobacillus sakei ob4.1 was more resistant to H<sub>2</sub>O<sub>2</sub> than *L. sakei* DSM 20017. *L. sakei* ob4.1 was isolated from one participant from obesity group. For comparison, *L. sakei* DSM 20017 (purchased from KCTC) was used as a reference strain and *L. Rhamnosus* GG (ATCC53103) (purchased from ATCC) was used as a negative control. (A) Survival rate was calculated by counting the number of viable cells of *L. sakei* strain in MRS for 1 h in 0, 5, 10, 15, and 30 mM H<sub>2</sub>O<sub>2</sub>. (B) Serial dilutions of bacterial cells were inoculated onto MRS plates. (C) Overnight-grown cultures of *L. sakei* ob4.1 or DSM 20017 were diluted in MRS medium (1:1000) and incubated at 37 °C, either aerobically by using shaking incubator (200 rpm) or anaerobically in anaerobic jar with GasPak (BD). CFUs were determined by measuring optical density at 600 nm every 4 h for 3 days. (D) Approximately 10<sup>6</sup> CFU/mL of bacterial suspension were added to a

monolayer of Caco-2 cells, with or without 0.5% H<sub>2</sub>O<sub>2</sub>, and incubated at 37 °C for 1 h. Non-adherent bacteria were removed, the number of viable bacterial cells was determined by spread plate method on MRS, and then incubated at 37 °C for 48 h. Adhesion ability was calculated by the percentage of adhered cells with respect to the total number of bacteria. Bars represent geometric mean ± standard deviation. \*\*, P < 0.01; \*\*\*, P < 0.001.

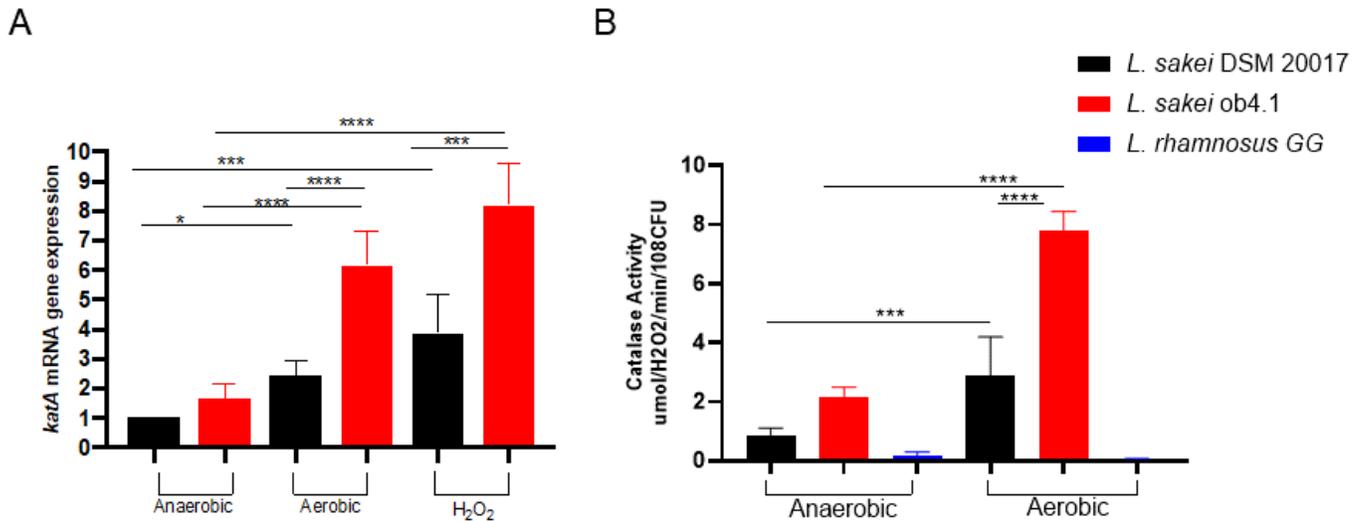


Figure 3.

### Figure 3

Oxidative stress increased *katA* gene expression and catalase activity in *Lactobacillus sakei* ob4.1. *L. sakei* ob4.1 and DSM 20017 were cultivated in MRS medium with 30 μM haematin, either aerobically using shaking incubator (200 rpm), anaerobically in anaerobic jar with GasPak (BD), or with 0.2 mM/L of H<sub>2</sub>O<sub>2</sub>, for 24 h at 37 °C; exponential-phase (OD<sub>600</sub> = 0.5) cells were used for analysis. (A) Transcript levels of *katA* were determined by quantitative real-time PCR using RNA isolated from indicated *Lactobacillus* strains. (B) Approximately 108 CFU/mL of cells were mixed with 0.8 mM of hydrogen peroxide and dichromate in acetic acid, followed by the measurement of absorbance at 570 nm. Catalase activity was expressed as μM H<sub>2</sub>O<sub>2</sub> degraded per minute per 108 CFU. Bars represent geometric mean ± standard deviation. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001.

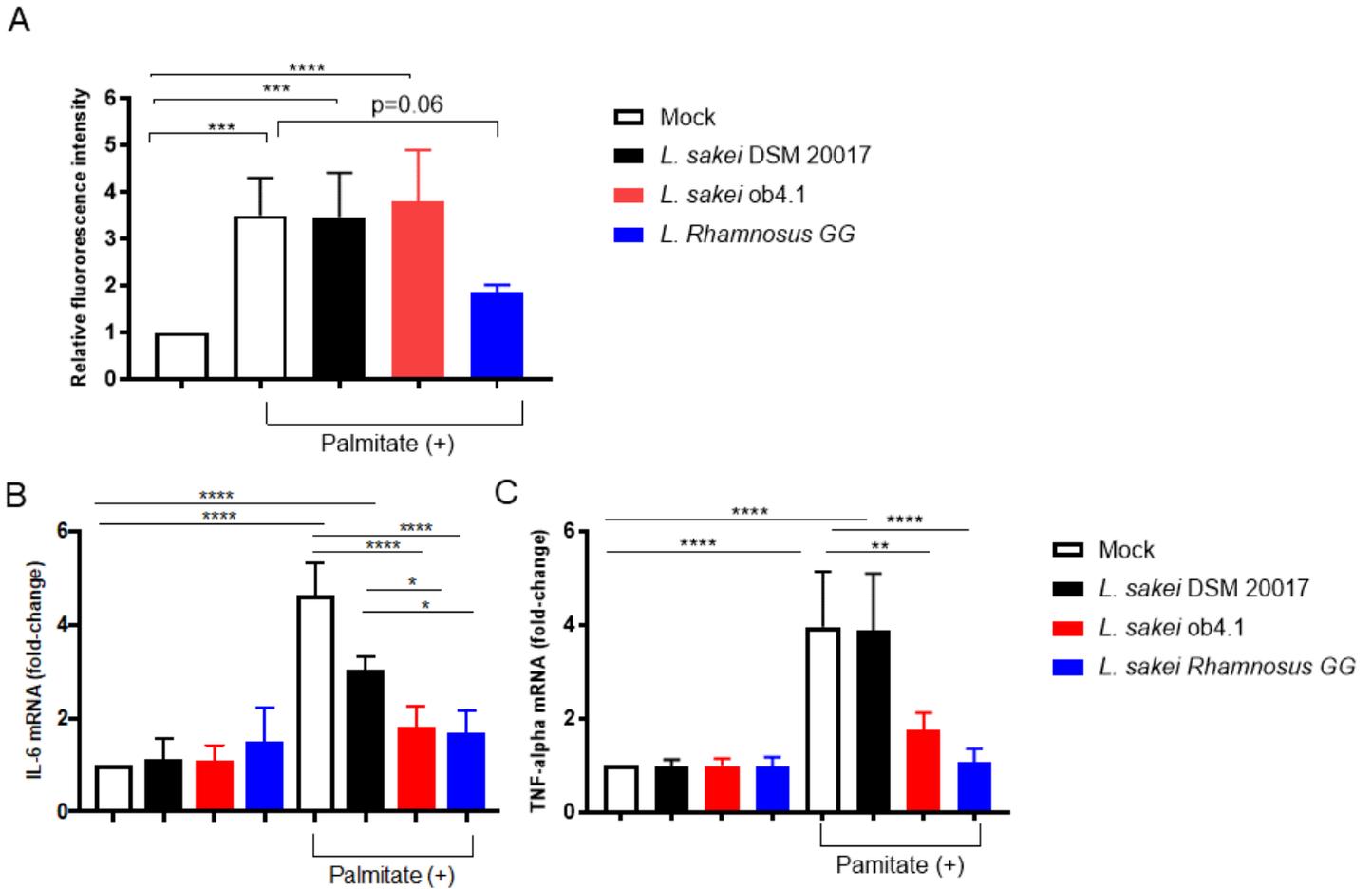


Figure 4.

#### Figure 4

Lactobacillus sakei ob4.1 decreased saturated fat-induced inflammation, not ROS, in Caco-2 cells. Differentiated Caco-2 cells were treated with palmitate (5 mM) or fatty acid bovine serum albumin (mock), and incubated at 37 °C and 5% CO<sub>2</sub> for 6 h; suspensions of Lactobacillus strains containing 10<sup>8</sup> CFU/mL cells were added and incubated for another 4 h. (A) ROS production was determined by measuring ROS-induced fluorescence intensity. Transcript levels of (B) IL-6 and (C) TNF-alpha were determined by quantitative real-time PCR from RNA isolated from Caco-2 cells treated with live indicative *L. sakei* strains. Bars represent geometric mean ± standard deviation. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001.

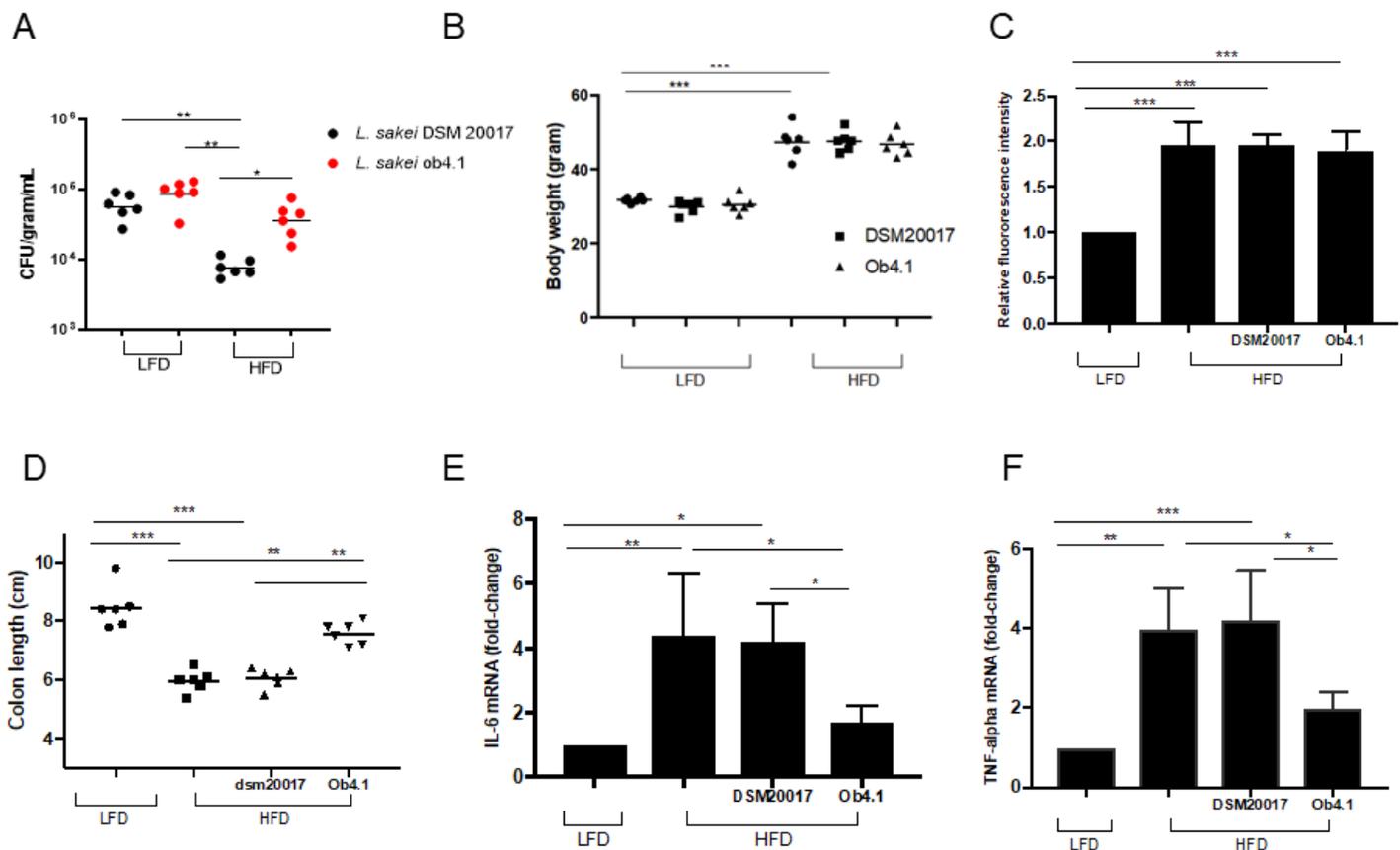


Figure 5.

## Figure 5

Lactobacillus sakei ob4.1 colonised more in mice on high-fat diet and restored high-fat diet-induced colon inflammation. Groups of male mice (N = 6) were reared on a high-fat diet (HFD, 60% fat) or a low-fat diet (LFD, 10% fat) for the whole experiment. (A) Two weeks after starting the diet, mice were mock-treated or inoculated with an indicative *L. sakei* strain ( $1 \times 10^9$  CFU/mice) and the number of *L. sakei* in faecal samples was determined two days after inoculation. (B–F) Two weeks after starting diet, mice were mock-treated or inoculated with an indicative *L. sakei* strain ( $1 \times 10^9$  CFU/mice/day) for 4 weeks. Mouse body weight (B) and colon length (D) were determined during necropsy. (C) ROS production was measured by measuring ROS-induced fluorescence intensity in mouse colon tissue. Transcript levels of (E) IL-6 and (F) TNF-alpha were determined by quantitative real-time PCR using RNA isolated from colon tissue. Dots represent data from individual animals and bars represent geometric mean (A, B, D). Bars represent geometric mean  $\pm$  standard deviation (C, E, F). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

## Supplementary Files

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