Effect of heat-killed \textit{Streptococcus thermophilus} on type 2 diabetes rats

Xiangyang Gao \textsuperscript{1} Equal first author, Fei Wang \textsuperscript{1} Equal first author, Peng Zhao \textsuperscript{1, 2}, Rong Zhang \textsuperscript{1}, Qiang Zeng \textsuperscript{Corresp. 1}

\textsuperscript{1} Health Management Institute, The Second Medical Center of Chinese PLA General Hospital, Beijing, China
\textsuperscript{2} Health Management Center, HangZhou Special Service Convalescent Center of Air Force, PLA, Hangzhou, China

Corresponding Author: Qiang Zeng
Email address: zengqianghospital@126.com

Background and Aims: The link between gut microbiota and type 2 diabetes (T2D) has been addressed by numerous studies. \textit{Streptococcus thermophilus} from fermented milk products, has been used as a probiotic in previous research. However, whether heat-killed \textit{S. thermophilus} can improve the glycaemic parameters of diabetic rats remains unanswered. In this study, we evaluated the effect of heat-killed \textit{S. thermophilus} on T2D model rats and the potential mechanisms of the effect.

Methods: Zucker diabetic fatty (ZDF) rats were used to generate a diabetic rat model induced by feeding a high-fat diet. Heat-killed \textit{S. thermophilus} were orally administered to normal and diabetic rats for 12 weeks. Intestinal microbiota analysis, histology analysis, oral glucose tolerance test (OGTT) and measurement of inflammatory factors were performed.

Results: We found that heat-killed \textit{S. thermophilus} treatment reduced fasting blood glucose levels and alleviated glucose intolerance and total cholesterol in diabetic ZDF rats. Additionally, heat-killed \textit{S. thermophilus} increased the IL-10 while reducing the levels of LPS, IL-6 and TNF-α in diabetic ZDF rats. The heat-killed \textit{S. thermophilus} treatment can normalize the structure of the intestinal and colon mucosal layer of diabetic rats. The characteristics of the gut microbiota in heat-killed \textit{S. thermophilus}-treated and control rats were similar. At the genus level, the abundances of beneficial bacteria, including Ruminococcaceae, Veillonella, Coprococcus and Bamesiella, were all significantly elevated by heat-killed \textit{S. thermophilus} treatment in ZDF diabetic rats.

Conclusion: Our study supports the hypothesis that treatment with heat-killed \textit{S. thermophilus} could effectively improve glycaemic parameters in T2D model rats. In addition, the potential mechanisms underlying the protection maybe include changing the composition of gut microbiota, reinforcing the intestinal epithelial barrier and the immunity of the intestinal mucosa, decreasing the level of inflammation, and then reducing the insulin resistance.
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Xiangyang Gao¹*, Fei Wang¹*, Peng Zhao¹,², Rong Zhang¹, Qiang Zeng¹#

¹Health Management Institute, The Second Medical Center of Chinese PLA General Hospital, Beijing, China.
²Health Management Center, HangZhou Special Service Convalescent Center of Air Force, PLA, HangZhou, China.
*These authors contributed equally to this work.
#Corresponding author:
Qiang Zeng
No.28, Fuxing road, Beijing, 100039, China.
Email address: zengqianghospital@126.com.
Abstract

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INTRODUCTION

Diabetes is a chronic metabolic disease and an important cause of mortality and morbidity worldwide, the prevalence of which is dramatically increasing. The number of adults with diabetes, mostly Type 2 diabetes (T2D), has increased to 422 million around the world. Diabetes and its complications account for more than 2 million deaths every year.

Recently, immense evidence has been obtained linking T2D and gut microbiota. The significant correlations with specific gut microbes, bacterial genes, and metabolic pathways in T2D patients were showed by a human metagenome-wide association study. The data from animal and human models also suggest that T2D is associated with a moderate degree to profound gut microbial dysbiosis. Increasing evidence indicates that gut microbiota are strongly associated with diabetes development. Other studies even show that gut microbiota markedly contribute to the incidence of T2D. The dysbiosis of gut microbiota may damage the intestinal epithelial barrier, and increase the intestinal permeability, and thus promotes metabolic endotoxemia, and systemic inflammation, leading to the development of insulin resistance.
thereby increasing the risk of developing T2D. These studies suggest that the gut microbiota are potential targets for the treatment of T2D.

Probiotics have been proven to be effective in T2D. Administration of probiotics in a rat model effectively inhibited gluconeogenesis in T2D. Treatments with probiotics have been demonstrated to be efficacious against tissue inflammation, and insulin resistance by modulating the gut microbial structure. However the efficacy in T2D subjects varies, depending on the types and strains of probiotics.

Probiotics, as defined by the World Health Organization (WHO), are live microorganisms, that confer a health benefit to the host, when administered in adequate amounts. However, in many cases, probiotic preparations comprised of dead cells and their metabolites can also exert a biological response similar to that seen with live cells. For example, both live and heat-killed Lactobacillus GG had a similar anti-inflammatory effect.

*Streptococcus thermophilus* is classified as a lactic acid bacterium (LAB), and it is found in fermented milk products, and generally used in the dairy industry. *S. thermophilus* scavenges reactive oxygen radicals, thus demonstrating its antioxidant properties. *S. thermophilus* also shows immunomodulatory effects by stimulating the gut immune system. And *S. thermophilus* has been used as a probiotic to help prevent developing insulin resistance in previous research. However, to our knowledge, the question as to whether heat-killed *S. thermophilus* can improve glycaemic parameters remains unanswered. In addition, the potential mechanisms underlying the possible protection are still poorly understood. Therefore, the purpose of this research was to identify the beneficial effects of heat-killed *S. thermophilus* on diabetic rats and the potential mechanisms.

**MATERIALS AND METHODS**

**T2D animal model**

The Zucker diabetic fatty (ZDF) rats were used as a T2D model. ZDF rats have been an important model for studying the mechanism of treatment on T2D. Seven-week-old male ZDF rats were purchased from Charles River (Beijing, China). After 1 week of acclimation, diabetes was then induced by feeding a high-fat diet of Purina5008 (17% kcal fat and 26.5% kcal protein, IPS Supplies, London, UK) for one month. Then, 12-week-old male ZDF rats were obtained, and fasting blood glucose (FBG) >11.1mmol/L was determined to be the standard concentration for the T2D model.

**Control rats**

Seven-week-old male Sprague-Dawley (SD) rats also were obtained from Charles River (Beijing, China). After acclimating for 1 week, they were used as control rats.

Both the ZDF and SD rats were maintained at 22±2°C with lights in an air-conditioned room with a 12-hour light/dark cycle, and were given free access to food and water. A standardized diet (kcal%: 10% fat, 20% protein, and 70% carbohydrate) was administered. All of the
experimental protocols were approved by the Animal Care Committee of the General PLA Hospital Animal Ethics Committee (Project CPLAGHAE-20171228-01).

Study design
The diabetic ZDF rats were randomly divided into two groups: a heat-killed *S. thermophilus*-treated diabetic group (DM + ST, KAWAI, Kawai Lactic Acid Bacteria Research Institute Co., Ltd., Japan, orally administered 0.21 g Kawai powder/kg body weight/day, n=5) and an untreated diabetic group (DM, orally administered the same volume of normal saline, n=5). Kawai powder contains 28.75% heat-killed *S. thermophilus* and 20.60% resistant dextrin, 20.00% isomaltooligosaccharide, 17.00% microcrystalline cellulose, 10.00% xylo-oligosaccharides, 2.55% Saccharomyces cerevisiae, and 1.10% lemon juice powder.

Control rats were randomly divided into an untreated control group (CON, administered normal saline, n=5) and a heat-killed *S.thermophilus*-treated control group (CON + ST, orally administered 0.21 g Kawai powder/kg body weight/day, n=5). After treatment for 12 weeks, fresh stool samples were obtained by stimulating the anus, and they were frozen and stored at -80°C for subsequent analysis. After food deprivation for 12 h, the rats were anesthetized, blood samples were collected from the aorta abdominalis, and then the rats were sacrificed.

Tissue collection and histology analysis
After rats were killed, the tissues of the ileum and colon were immediately excised, and then were cleaned with ice-cold phosphate-buffered saline solution. The tissues were fixed in 4% formalin solution, then embedded in paraffin before being cut into 4-μm slices, followed by hematoxylin-eosin (H&E) staining for measurement of villi length and crypt depth (10 villi and 10 crypts per section) under a light microscope (SZX16, Olympus, Japan).

Western blot analysis
The ileum and colon tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktail (Roche,Indianapolis, IN, USA). Protein homogenates were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking for 1 h with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween (TBST: 50mM Tris-HCl, 150mM NaCl, 0.1% Tween 20,pH 7.4), the membranes were incubated overnight with specific primary antibodies against Occludin(Abcam, Cambridge,UK), ZO-1 (Zonula occludens) (Santa Cruz Biotechnology, CA, USA), and β-actin (Zsbio, Beijing, China) at 4°C. Then, the membranes were incubated for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse IgG-HRP) (Jackson ImmunoResearch Inc, West Grove, PA, USA), and the bands were detected by using enhanced chemiluminescence. The blots were scanned by a Bio-Rad ChemiDoc XRS and the intensity of each protein was quantified by Gel Image system V4.00 software (Tanon, Shanghai, China).

Oral glucose tolerance test (OGTT)
At the end of the trial, an OGTT was performed after fasting for 12 h. Glucose (2 g/kg body weight) was orally administered to the rats. The blood glucose levels which were obtained from the tail were recorded with a OneTouch UltraEasy glucometer (Johnson & Johnson, New Brunswick, NJ, USA) before and 15, 30, 60, 90, and 120 min after the glucose load. The area under the curve (AUC) was calculated by using the linear trapezoid method.

**Measurement of inflammatory factors, serum insulin, lipid profile, HOMA-IR, and HbA1c**

After food deprivation for 12 h, rat serum was obtained to analyze inflammatory factors (interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS) (ELISA, Elabscience, Wuhan, China), insulin (ELISA, Millipore, Billerica, MA, USA), total cholesterol (TC), triglyceride (TG, oxidase method; InTec Products, Fujian, China), high-density lipoprotein cholesterol concentrations (HDL-C), and low-density lipoprotein cholesterol concentrations (LDL-C, direct method, InTec Products, Fujian, China), according to the manufacturer's instruction. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the following formula: fasting blood glucose (FBG, mmol/L) × fasting serum insulin (μIU/ml)/22.5. Rat plasma was also analyzed for HbA1c (Immunoturbidimetry, InTec Products, Fujian, China).

**Intestinal microbiota analysis**

DNA extractions from total fecal bacteria were obtained using a QIAamp Stool DNA Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The microbial 16S rRNA hypervariable regions V3-V4 were amplified with indexes and adaptor-linked universal primers (341F: 50-ACTCCTACGGGAGGCAGCAG-30, 806R: 50-GGACTACHVGGGTWTCTAA-30T). PCR was performed by using a KAPA HiFi Hotstart PCR kit (KAPA Biosystems, Wilmington, DE, USA) with high fidelity enzyme in triplicate. Amplicon libraries were quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and then sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) for 250-bp paired-end reads. After discarding the singletons and removing chimeras, operational taxonomic units (OTUs) were generated using USEARCH (v7.0.1090) at 97% similarity by clustering the tags. Final OTUs were taxonomically classified based on the RDP classifier version 2.2 algorithm using the GreenGene database. Alpha diversity (Chao1, Shannon, Simpson) and beta diversity (principal coordinates analysis (PCoA) plots) were analyzed using QIIME version 1.7.0. In addition, a t test was performed to compare the differences between groups by using STAMP. The relative abundance of bacteria is expressed as the percentage (%).

**Data analysis**

The data are expressed as the mean ± standard deviation (SD). When the data were normal and variances were equal, differences among the groups were analyzed using t test. For non-normal distribution data, ln transformation was carried out before analysis. A p value <0.05 was
considered statistically significant. All of the statistical analyses were performed using the Statistical Package for Social Sciences version 17 software (SPSS Inc., Chicago, IL, USA).

Results

Body weight
The body weights of the heat-killed *S. thermophilus*–treated diabetic rats were comparable with those of the untreated diabetic rats (P > 0.05, Table 1). There was also no significant difference in body weight between the CON group and CON+ST group (P > 0.05, Table 2).

Fasting blood glucose level and glucose tolerance
The heat-killed *S. thermophilus* treatment reduced fasting blood glucose (FBG) levels in diabetic rats (P < 0.05, Figure 1). The blood glucose levels significantly decreased before and 15, 60, and 90 min after glucose load (P < 0.05, Figure 1) in the DM+ST group as compared to those in the DM group according to the OGTT. At the time points of 30 and 120 min after the glucose load, the blood glucose levels were lower in the DM+ST group than those in DM group, but the differences were not significant (P > 0.05, Figure 1). Compared with the DM group, the glucose area under the curve (AUC) for the OGTT in the DM+ST group exhibited a reduced glucose AUC by 14.7% (P < 0.05, Figure 2).

According to the OGTT, although there were no significant differences before and 15, and 120 min after the glucose load between the CON group and CON+ST group, the blood glucose levels significantly decreased 30 and 60 min after the glucose load (P < 0.05, Figure 3) in the CON+ST group as compared to those in the CON group. The CON+ST group exhibited a reduced glucose AUC by 18.2% (P < 0.05, Figure 4) for the OGTT, compared with the CON group.

Fasting insulin, HbA1c, and HOMA-IR
The heat-killed *S. thermophilus* treatment reduced serum insulin levels, HbA1c, and HOMA-IR (P < 0.05, Table 1) in ZDF diabetic rats. However, compared to the CON group, the heat-killed *S. thermophilus* treatment rats failed to produce significantly lower serum insulin levels or HOMA-IR in the CON+ST group (P < 0.05, Table 2).

Serum biochemical parameters
There were no significant differences in the serum creatinine, alanine aminotransferase (ALT), carbamide, or uric acid levels between the two groups in ZDF diabetic rats. The level of TC significantly increased in the DM+ST group, while the heat-killed *S. thermophilus* treatment did not significantly reduce the TG, HDL-C, or LDL-C levels in diabetic rats (P < 0.05, Table 1). In contrast, there were no significant difference in serum biochemical parameters in the CON+ST group compared to the CON group (P > 0.05, Table 1).

Inflammatory factors
Compared with the DM group, the inflammatory factors LPS, IL-6, and TNF-α significantly decreased and IL-10 significantly increased in the DM+ST group (P < 0.05, Table 1). There were no significant differences in the inflammatory factors between the CON+ST group and CON group (P > 0.05, Table 2).

**Histological analysis**
We examined the heat-killed *S. thermophilus* effects on the villi length and crypt depth in the ileum. In the diabetic rats, the intestinal mucosal layer was characterized by disturbed mucosal architecture, shortened villi, blunted villus tips, and inflammatory cell infiltration. In the DM+ST group, oral administration of *S. thermophilus* restored the normal structure of the intestinal mucosal layer (Figure 5). The length of villi and depth of crypts in the DM+ST group were significantly increased compared to those in DM group (Figure 6). Additionally, goblet cells were counted per villus/crypt in the ileum. The ileum exhibited a significant increase in total goblet cell number after treatment with *S. thermophilus* (39.2±4.2 vs 20.9±5.0, p < 0.05). Similar findings were also seen in the colonic tissues (Figure 5 and 7). There were no differences in the villi length and crypt depth and the numbers of goblet cells between the CON and CON+ST groups (Figure 8).

**Western blot analysis**
To explore the mechanisms underlying the heat-killed *S. thermophilus* effects on the barrier function, the expression levels of Occludin and ZO-1 proteins were determined by Western blot analysis. The results showed that Occludin and ZO-1 proteins in the DM+ST group were significantly elevated compared with the DM group both in the ileum and colon tissues (1.76-fold increases for Occludin and 2.29-fold increases for ZO-1 in the ileum tissues; 1.64-fold increases for Occludin and 1.46-fold increases for ZO-1 in the colon tissues, vs. the DM group) (Figure 9). There were no differences in the expression levels of Occludin and ZO-1 in the ileum or colon tissues between the CON and CON+ST groups (Figure 10).

**Characterization of gut microbiota**
In ZDF diabetic rats, the richness of the gut microbiota was increased in the DM+ST group compared with the DM group; however, the difference was not significant, as shown in Table 3. Significant difference did not exist between the CON+ST group and the CON group. As shown in Fig.11, to assess the bacterial community between two groups, a principal coordinate analysis (PCoA) for the unweighted UniFrac distance matrices was performed. The first two principal coordinates of PCoA (components 1 and 2) were separated into DM+ST and DM groups, which shared overlapping regions. As in the above analysis, the DM+ST and DM groups, and COM+ST and CON groups exhibited similar alpha and beta diversities in the gut microbiota. The results indicate that the treatment with heat-killed *S. thermophilus* could not improve the richness of the gut microbiota.
At the genus level, the abundance of *Ruminococcaceae*, *Veillonella*, *Coprococcus*, and *Barnesiella* was significantly elevated by heat-killed *S. thermophilus* treatment in ZDF diabetic rats (P < 0.05, Fig. 12), whereas *Phascolarctobacterium* and *Dorea* abundances were reduced by heat-killed *S. thermophilus* treatment in SD control rats (P < 0.05, Fig. 13).

**DISCUSSION**

In this study, heat-killed *S. thermophilus* bacteria were administered to ZDF T2D rats to test whether they have a protective effect. The ZDF diabetic rat is a well-characterized model of T2D, and the rats has been used in many studies to examine human T2D pathophysiology and the effects of therapeutic options. Interestingly, we found that the heat-killed *S. thermophilus* treatment effectively moderated insulin resistance and glucose intolerance in the ZDF T2D rat model. To our knowledge, this is the first report about the effect of the heat-killed *S. thermophilus* treatment on glycaemic parameters of diabetic rats. Many previous studies focused on the relation between live *S. thermophilus* and human. For instance, a multispecies probiotic supplement consisting of *S. thermophilus* reduced the fasting plasma glucose and serum high-sensitivity C-reactive protein, and increased plasma total glutathione. And the probiotic mix VSL#3, which contains *S. thermophilus*, increased insulin sensitivity, and affected the composition of gut microbiota. So our work provides new insights into the function of the heat-killed *S. thermophilus*.

Another effect of the heat-killed *S. thermophilus* treatment is that of significantly reducing the level of TC in the diabetic rats used in this study. The effect of removing cholesterol probably occurs by two mechanisms: binding cholesterol to the cell surface or deconjugating bile salts to prevent their recycling.

In addition, we found that the heat-killed *S. thermophilus* treatment increased the abundance of *Ruminococcaceae*, *Veillonella*, *Coprococcus* and *Barnesiella* at the genus level in diabetic rats. The normal gut microbiota has many functions, such as protection against pathogens, immunomodulation, maintenance of the gut mucosal barrier structural integrity, and nutrient and drug metabolism. As a member of short chain fatty acid producers, *Ruminococcaceae* is inversely correlated with increased intestinal permeability, and alcoholic cirrhosis. The abundance of *Ruminococcaceae* has been observed to significantly increase after treatment with fucoidan. *Coprococcus* is a butyrate-producing genera. Dietary intervention including extensively hydrolyzed casein formula supplemented with *Lactobacillus rhamnosus GG* to enrich Coprococcus could accelerate tolerance acquisition in infants who are allergic to milk. *Veillonella* are normal bacteria found in the intestines of mammals, that are well known for their lactate fermenting abilities. A positive association has been found between lactose levels and the abundance of the *Veillonella* genus. Anaerobic bacteria belonging to the *Barnesiella* genus enable clearance of intestinal colonization by the highly antibiotic-resistant bacterium vancomycin-resistant Enterococcus. When compared with high-fat, high-sucrose-fed mice, *Barnesiella spp* are the main discriminative feature of chow-fed mice. Therefore, *Barnesiella* may have a beneficial impact on host metabolism.
Many effects of probiotics are mediated through immune regulation and through the balance of anti-inflammatory and pro-inflammatory cytokines. In this study, the heat-killed \textit{S. thermophilus} treatment significantly decreased the inflammatory factors LPS, IL-6, and TNF-α, and increased IL-10. From the membranes of gram-negative bacteria, LPS penetrates into the blood via impaired permeability of the intestinal mucosa, which is caused by the reduced expression of adhesion and tight junction proteins\textsuperscript{50}. Then, LPS triggers a strong pro-inflammatory reaction and secretion of proinflammatory cytokines from the host cells, followed by metabolic endotoxemia\textsuperscript{51}. Metabolic endotoxemia increases systemic inflammation and impairs insulin sensitivity in both adipose tissue and the liver\textsuperscript{52}. It can also impair insulin signaling by inducing endoplasmic reticulum stress and the activity of a histone acetyltransferase\textsuperscript{53}. The high circulating LPS characterizes both incident and prevalent diabetes in a clinical observation also suggests the relevance of this putative mechanism to humans\textsuperscript{54}. As the product of pro-inflammatory cells, IL-6 is involved in many biological processes, such as the host response to acute-phase reactions, haematopoiesis, enteric pathogens, and terminal differentiation of B-lymphocytes\textsuperscript{55}. IL-10 is a potent deactivator of macrophage/monocyte proinflammatory cytokine synthesis\textsuperscript{56}, such as downregulation of TNF-α secretion by macrophages\textsuperscript{57}.

It was also found that the heat-killed \textit{S. thermophilus} treatment protected the intestinal barrier. In our study, an increased ileum villus/crypt length and number of goblet cells were observed in the DM+ST group with \textit{S. thermophilus} administration compared with the DM group. This is consistent with previous studies reporting that probiotic administration markedly deepened jejunal crypts in healthy rats\textsuperscript{58}, and both villus and crypt were lengthened after treatment by emu oil\textsuperscript{59}. The main role of goblet cells is to protect the mucous membrane by secreting mucus\textsuperscript{60}. There is a strong association between intestinal flora and secretion of mucin\textsuperscript{61}, as goblet cells may be regulated by interactions between the gastrointestinal mucosa and specific bacterial peptides\textsuperscript{62}. The results of our study also showed that the Occludin and ZO-1 proteins in the DM+ST group were significantly elevated compared with the DM group both in the ileum and colon tissues. Intestinal barrier integrity is maintained by the tight junctions those are made of transmembrane, scaffold and adaptor proteins. Occludin is transmembrane protein embedded in the intracellular actin through attachment to adaptor protein ZO-1\textsuperscript{63}. It is widely reported that commensal bacteria have profound effects on epithelial integrity and permeability, particularly, on tight junctions maintenance\textsuperscript{64}. A dysbiosis adversely enhances intestinal permeability by modulating the expression of epithelial tight junction proteins ZO-1 and Occludin\textsuperscript{65}. The mucosal barrier is very important for protecting the host tissue from damage that is mediated by toxic products or luminal pathogens obtained from food or pathogenic bacteria, while allowing uptake of nutrients at the same time. A previous study showed that feeding fermented milk produced by \textit{S. thermophilus} and \textit{Bifidobacterium breve} could reinforce the intestinal barrier\textsuperscript{66}. Another study also showed that live \textit{S. thermophilus} significantly increased the transepithelial electrical resistance in the intestinal Caco-2 cell monolayer by enhancement.
(actinin, occludin) or maintenance (actin, ZO-1) of cytoskeletal and tight junctional protein phosphorylation. In the current study, the *S. thermophilus* used was heat-killed instead of live cells. Both live and dead cells are capable of generating a biological response. Our result is consistent with a recent study which shows that pasteurized *Akkermansia muciniphila* is able to ameliorate high-fat diet induced dysglycemia. In a meta-analysis, modified (heat-killed or sonicated) probiotics were found to have effects similar to those of the living probiotics in most trials. The effects of heat-killed probiotics may be attributed to the dead cells and/or their metabolites. For example, metabolites released by *S. thermophilus* exerted an anti-TNF-α effect and were capable of crossing the intestinal barrier. Besides, a recombinant protein isolated from the *A. muciniphila* membrane can lead to an improved gut barrier. Notably, even *A. muciniphila*-derived extracellular vesicles can decrease gut permeability by regulating the tight junctions. It has also been documented that bacterial muramyl dipeptide reduces inflammation and promotes insulin signaling in the state of metabolic endotoxemia, and glycemias. As a bacterial metabolite, indole is able to counteract the pro-inflammatory and metabolism-altering effects of LPS in the liver. Similarly, SCFAs can improve barrier function, decrease inflammation, and promote the metabolism of lipids and glucose. In addition, microbiota-derived succinate can also improve glucose metabolism by acting on intestinal gluconeogenesis.

One limitation to widespread use of probiotic therapy is the concern regarding adverse effects, which may cause some pathology of their own. Compared with live probiotics, heat-killed probiotics are safer for purposes such as application in immunosuppressed patients and children. Another problem with live probiotics is that they would have to survive proteolytic enzymes and the low pH of stomach acid. The recovery rate of total *S. thermophilus* from the terminal ileum of minipigs was very low after digesting a certain amount of live cells. The preparation and administration of heat-killed probiotics are convenient compared to live probiotics. Products based on dead cells are easier to standardize, and store, and they also have a long shelf-life. Therefore, heat-killed probiotics may be a promising and safer alternative to live probiotics.

After the analysis of numerous studies, it was proposed that there may be a bacteria mucosal immunity-inflammation-diabetes (BMID) axis, through which herbal monomers and formulae improve diabetes. In this study, heat-killed *S. thermophilus* may also affect diabetes through the BMID axis by increasing the abundance of beneficial bacteria, protecting the intestinal epithelial barrier, and suppressing IL-6, LPS, and TNF-α secretion, and the end result is moderation of insulin tolerance.

**CONCLUSION**

Our study supports the hypothesis that treatment with heat-killed *S. thermophilus* could effectively improve the glycaemic parameters of T2D model rats. In addition, the potential mechanisms underlying the protection may consist of changing the composition of gut
microbiota, reinforcing the intestinal epithelial barrier and the immunity of the intestinal mucosa, decreasing the level of inflammation, and then reducing insulin resistance.

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**Table 1** (on next page)

The differences in some variables between the DM+ST and DM groups

N=5 in each group. Data represented as means ± SD. ALT: Alanine aminotransferase, TC: Total cholesterol, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, LNIL6: Ln transformation of interleukin-6, LNIL10: Ln transformation of interleukin-10, LnTNF-α: Ln transformation of tumornecrosisfactor-α, LPS: Lipopolysaccharide, HOMA-IR: Homeostasis model assessment of insulin resistance, FBG: Fasting blood glucose.

The heat-killed *S. thermophilus* treatment reduced TC, LPS, IL-6, IL-10, TNF-α, fasting insulin levels, HbA1c, FBG, and HOMA-IR in ZDF diabetic rats; *P < 0.05.*
| Variables                  | DM    | DM+ST | t     | p    |
|----------------------------|-------|-------|-------|------|
| Weight (g)                 | 354.2±35.2 | 360.2±33.0 | -0.3  | 0.788 |
| Creatinine (umol/l)        | 16.0±6.2   | 15.0±2.1   | 0.3   | 0.742 |
| ALT (U/L)                  | 119.4±51.5 | 123.4±52.7 | -0.1  | 0.906 |
| Carbohydrate (mmol/l)      | 6.0±0.8    | 7.2±1.6    | -1.6  | 0.159 |
| Uric acid (umol/l)         | 116.2±32.6 | 122.6±18.2 | -0.4  | 0.711 |
| TC (mmol/l)                | 5.5±0.4    | 4.7±0.2    | 4.1   | 0.003*|
| Triglyceride (mmol/l)      | 3.0±0.9    | 2.5±0.3    | 1.2   | 0.304 |
| HDL-C (mmol/l)             | 2.7±0.2    | 2.5±0.2    | 1.8   | 0.115 |
| LDL-C (mmol/l)             | 1.2±0.2    | 0.9±0.1    | 2.5   | 0.063 |
| LPS (ng/ml)                | 0.7±0.1    | 0.5±0.1    | 2.9   | 0.019*|
| LNIL6 (pg/ml)              | 4.6±0.6    | 3.9±0.3    | 2.7   | 0.038*|
| LNIL10 (pg/ml)             | 4.0±0.3    | 4.5±0.3    | -2.4  | 0.046*|
| LnTNF-α (pg/ml)            | 4.1±0.3    | 3.6±0.2    | 3.0   | 0.017*|
| Fasting insulin (uIU/ml)   | 107.6±18.1 | 67.0±8.3   | 4.6   | 0.002*|
| HOMA-IR                    | 106.7±25.5 | 40.8±3.8   | 5.7   | 0.004*|
| HbA1c (%)                  | 12.0±2.0   | 8.6±1.2    | 3.3   | 0.011*|
| FBG (mmol/L)               | 22.3±3.6   | 13.7±1.0   | 3.1   | 0.036*|
The differences in some variables between the CON+ST and CON groups

\( N=5 \) in each group. Data represented as means ± SD. ALT: Alanine aminotransferase, TC: Total cholesterol, HDL-C: High-density lipoproteincholesterol, LDL-C: Low-density lipoproteincholesterol, LNIL6: Ln transformation of interleukin-6, LNIL10: Ln transformation of interleukin-10, LnTNF-α: Ln transformation of tumornecrosisfactor-α, LPS: Lipopolysaccharide, HOMA-IR: Homeostasis model assessment of insulin resistance, FBG: Fasting blood glucose. Compared to the CON group, the heat-killed S. thermophilus treatment rats failed to show significant variations.
| Variables               | CON+ST     | CON       | t   | p    |
|-------------------------|------------|-----------|-----|------|
| Weight(g)               | 503.2±48.5 | 476.8±37.3| 1.0 | 0.363|
| Creatinine(umol/l)      | 24.2±2.8   | 26.2±4.7  | -0.8| 0.437|
| ALT(U/L)                | 37.6±9.8   | 36.8±7.6  | 0.1 | 0.889|
| Carbamide(mmol/l)       | 6.1±1.4    | 5.7±1.5   | 0.4 | 0.709|
| Uric acid(umol/l)       | 122.8±21.7 | 154.0±66.6| -1.0| 0.348|
| TC(mmol/l)              | 1.4±0.3    | 1.8±0.4   | -1.8| 0.11 |
| Triglyceride (mmol/l)   | 0.7±0.2    | 0.7±0.3   | 0.1 | 0.963|
| HDL-C(mmol/l)           | 0.8±0.2    | 0.9±0.2   | -1.3| 0.246|
| LDL-C(mmol/l)           | 0.3±0.1    | 0.4±0.1   | -1.6| 0.16 |
| LPS(ng/ml)              | 0.4±0.1    | 0.4±0.1   | -1.0| 0.369|
| Inln6(pg/ml)            | 4.0±0.2    | 4.1±0.3   | -0.6| 0.585|
| InlnL10(pg/ml)          | 4.3±0.5    | 4.1±0.1   | 0.8 | 0.465|
| InTNF-α(pg/ml)          | 3.9±0.4    | 4.1±0.3   | -1.1| 0.323|
| Fasting insulin (uIU/ml)| 41.1±7.0   | 42.2±12.5 | -0.2| 0.863|
| FBG (mmol/L)            | 6.7±0.6    | 6.9±0.6   | -0.4| 0.72 |
| HOMA-IR                 | 12.3±2.8   | 12.7±2.9  | -0.2| 0.857|
Table 3 (on next page)

Alpha diversity indices

Data are presented as means ± SD (n = 5). In ZDF diabetic rats, the richness of the gut microbiota was increased in the DM+ST group compared with the DM group; however, the difference was not significant (P>0.05, t test and Wilcoxon rank-sum test). Significant difference did not exist between the CON+ST group and the CON group (P>0.05, t test and Wilcoxon rank-sum test).
|               | CON      | CON+ST   | DM       | DM+ST    |
|---------------|----------|----------|----------|----------|
| OTUs          | 1497.40 ± 327.41 | 1504.20 ± 275.38 | 1064.60 ± 230.90 | 1298.60 ± 323.53 |
| Chao1         | 4143.66 ± 490.57 | 4218.70 ± 524.60 | 3253.11 ± 518.16 | 3185.49 ± 733.41 |
| Shannon       | 117.34 ± 11.09  | 121.01 ± 11.65 | 86.96 ± 8.13 | 90.94 ± 20.40 |
| Simpson       | 6.76 ± 0.66     | 6.88 ± 0.39  | 4.92 ± 0.70 | 5.72 ± 1.22  |
| PD_whole_tree | 0.93 ± 0.04     | 0.93 ± 0.02  | 0.81 ± 0.08 | 0.87 ± 0.09  |
Figure 1 (on next page)

The effect of heat-killed S.thermophilus on blood glucose during the OGTT in the DM+ST and DM groups

OGTT: oral glucose tolerance test. Error bars represent one standard deviation. The blood glucose levels significantly decreased before and 15, 60, and 90 min after glucose load in the DM+ST group as compared to those in the DM group according to the OGTT. * p<0.05.
Area under the curve (AUC) for the OGTT in the DM+ST and DM groups

Compared with the DM group, the glucose area under the curve (AUC) for the OGTT in the DM+ST group exhibited a reduced glucose AUC by 14.7%; *P<0.05.
AUC (mmol/L/min)

DM
DM + ST

*
The effect of heat-killed S.thermophilus on blood glucose during the OGTT in the CON+ST and CON groups

OGTT: oral glucose tolerance test. Error bars represent one standard deviation. According to the OGTT, although there were no significant differences before and 15, and 120 min after the glucose load between the CON group and CON+ST group, the blood glucose levels significantly decreased 30 and 60 min after the glucose load in the CON+ST group as compared to those in the CON group. * p<0.05.
**Figure 4** (on next page)

Area under the curve (AUC) for the OGTT in the CON+ST and CON groups

The CON+ST group exhibited a reduced glucose AUC by 18.2% for the OGTT, compared with the CON group; *P<0.05.
Figure 5

Representative histology of the ileum and colon with HE stain in T2D model rats.

(A) Histology of the ileum in the DM group, (B) histology of the ileum in the DM+ST group, (C) histology of the colon in the DM group, and (D) histology of the colon in the DM+ST group. The image acquisition phase was performed with a 50X objective. Scale bar = 200 μm. In the diabetic rats, the intestinal mucosal layer was characterized by disturbed mucosal architecture, shortened villi, blunted villus tips, and inflammatory cell infiltration. In the DM+ST group, oral administration of *S. thermophilus* restored the normal structure of the intestinal mucosal layer.
The length of villi and depth of crypts in the ileum of diabetic rats

The length of villi and depth of crypts in the ileum in the DM+ST group were significantly increased compared to those in DM group; *P<0.05.
Figure 7 (on next page)

The length of villi and depth of crypts in the colon of diabetic rats

The length of villi and depth of crypts in the colon in the DM+ST group were significantly increased compared to those in DM group; *P<0.05.
Figure 8

Representative histology of the ileum and colon with HE stain in SD rats

(A) Histology of ileum in the CON group, (B) Histology of ileum in the CON+ST group, (C) Histology of colon in the CON group, (D) Histology of colon in the CON+ST group. The image acquisition phase was performed with a 50X objective. Scale bar = 200 μm. The characteristics of the intestinal mucosal layer were similar between the CON+ST group and CON group. There were no differences in the villi length and crypt depth and the numbers of goblet cells between the CON+ST group and CON group.
Figure 9 (on next page)

Effects of the heat-killed *S. thermophilus* treatment on tight junction proteins in the DM+ST and DM groups.

(A) Ileum and colon extracts from DM+ST and DM groups were used for Western blot analysis; (B) Expression levels of Occludin were quantified by measuring band densities; (C) Expression levels of ZO-1 were quantified by measuring band densities. β-actin was used as a loading control. * p<0.05.
Figure 10 (on next page)

Effects of the heat-killed *S. thermophilus* treatment on tight junction proteins in the CON+ST and CON groups

(A) Ileum and colon extracts from CON+ST and CON groups were used for Western blot analysis; (B) Expression levels of Occludin were quantified by measuring band densities; (C) Expression levels of ZO-1 were quantified by measuring band densities. β-actin was used as a loading control.
Figure 11

PCoA of unweighted UniFrac distances of the gut bacterial communities between the DM+ST and DM groups

The first two principal coordinates of PCoA were separated into DM+ST and DM groups, which shared overlapping regions.
Figure 12 (on next page)

The $t$ test results of the relative abundance (%) of bacteria from the DM+ST and DM groups.

At the genus level, the abundance of *Ruminococcaceae, Veillonella, Coprococcus, and Bamesiella* was significantly elevated by heat-killed *S. thermophilus* treatment in ZDF diabetic rats.
Figure 13(on next page)

The t test results of the relative abundance (%) of bacteria from the CON+ST and CON groups

Compared with the CON group, Phascolarctobacterium and Dorea abundances were reduced by heat-killed S. thermophilus treatment in the CON+ST group.
