Effects of *Lactobacillus delbrueckii* on Serum Lipid Parameters, Cholesterol Metabolism, and Enzyme Activities and Gene mRNA Expression Related to Fat Deposition in Finishing Pigs: Postprint

Authors: Rui Li, Hou Gaifeng, Wei Liangkai, Peng Wei, Pan Jie, Huang Xingguo

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Abstract

This study aimed to investigate the effects of dietary supplementation with Lactobacillus delbrueckii preparation on blood lipid parameters, hepatic cholesterol metabolism and fat deposition-related enzyme activities, tissue cholesterol metabolism and fat deposition-related gene mRNA expression, and subcutaneous adipose tissue morphology in fattening pigs. One hundred and twenty healthy "Duroc × (Landrace × Large White)" fattening pigs with an average body weight of $(65.34\pm3.64)kgwereselectedandrandomlydividedinto2groups,with6replicates(pens)pergroupand10pigsperr$ hydroxylase (CYP7A1) activity ($P<0.05$), and a highly significant decrease in adipose triglyceride lipase (ATGL) activity ($P<0.01$). 3) Compared with the control group, the experimental group exhibited a decreasing trend in the relative mRNA expression of ileal bile acid binding protein (IBABP) ($P=0.07$), a significant increase in the relative mRNA expression of peroxisome proliferator-activated receptor γ (PPARγ) in subcutaneous adipose tissue ($P<0.05$), and a decreasing trend in subcutaneous adipocyte diameter ($P=0.09$). These results suggest that Lactobacillus delbrueckii can regulate hepatic cholesterol and lipid metabolism by interfering with bile acid absorption in the ileum of fattening pigs.

Full Text

Effects of *Lactobacillus delbrueckii* on Serum Lipid Indexes, Enzyme Activities and Gene mRNA Expression Related to Cholesterol Metabolism and Fat Deposition in Finishing Pigs

LI Rui[1,2], HOU Gaifeng[1,2], WEI Liangkai[1,2], PENG Wei[1,2], PAN Jie[1,2], HUANG Xingguo[1,2]*

[1]College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, China
[2]Hunan Co-Innovation Center of Animal Production Safety, Changsha 410128, China

Abstract

This experiment was conducted to investigate the effects of dietary *Lactobacillus delbrueckii* preparation on serum lipid indexes, hepatic enzyme activities related to cholesterol metabolism and fat deposition, tissue mRNA expression of genes involved in cholesterol metabolism and fat deposition, and subcutaneous adipose tissue morphology in finishing pigs. One hundred and twenty healthy "Duroc × (Landrace × Yorkshire)" finishing pigs with an average body weight of $(65.34\$\pm3.64)kgwererandomlyallocatedintotwogroups, eachconsistingofsixreplicates(pens)withtenpigsperre$ hydroxylase (CYP7A1) activity was significantly increased (P<0.05), and adipose triglyceride lipase (ATGL) activity was significantly decreased (P<0.01) in the experimental group. 3) The mRNA relative expression of ileal bile acid binding protein (IBABP) tended to decrease (P=0.07), while the mRNA relative expression of peroxisome proliferator-activated receptor γ (PPARγ) in subcutaneous adipose tissue was significantly increased (P<0.05) in the experimental group. Subcutaneous adipocyte diameter also showed a decreasing trend compared with the control group (P=0.09). These results indicate that *L. delbrueckii* can regulate hepatic cholesterol and lipid metabolism by interfering with bile acid absorption in the ileum of finishing pigs.

Keywords: *Lactobacillus delbrueckii*; finishing pigs; enzyme activities; gene expression

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Cholesterol is widely distributed in various tissues and cells of the body and plays a crucial role in the structure and function of animal cell membranes, representing an essential nutrient for humans. However, long-term consumption of high-fat foods leads to excessive blood cholesterol or lipid levels, which can cause cardiovascular diseases. Currently, cardiovascular disease is the leading cause of

death worldwide [1]. The World Health Organization (WHO) predicts that approximately 23.6 million people will die from cardiovascular diseases globally by 2030 [2]. Research has shown that animal species, age, body weight, sex, and dietary factors all influence nutrient distribution in tissues, fatty acid composition, and cholesterol content in fat and muscle tissues [3-5]. Among these, dietary factors have received considerable attention from researchers. Studies have confirmed that dietary supplementation with lactic acid bacteria preparations can effectively reduce serum cholesterol levels in animals and regulate cholesterol and lipid metabolism [2,6-8]. Our previous research demonstrated that *Lactobacillus delbrueckii*, as a lactic acid bacteria preparation, can effectively reduce serum cholesterol content and improve blood lipid profiles in finishing pigs, although the specific mechanism of action remains unclear [7-8]. Numerous reports have addressed the cholesterol-lowering mechanisms of lactic acid bacteria; however, most studies have been based on high-fat animal models, which may not fully reflect normal physiological metabolism. Therefore, this experiment used finishing pigs with strong fat deposition capacity as the research model and fed them diets supplemented with *L. delbrueckii* preparation to explore its mechanisms of action on cholesterol metabolism and tissue fat deposition, providing a scientific basis for the development of low-cholesterol pork.

1. Materials and Methods

1.1 Experimental Material

Lactobacillus delbrueckii was screened by the Functional Microbiology Laboratory of the College of Animal Science and Technology at Hunan Agricultural University and identified and preserved by the China Center for Type Culture Collection at Wuhan University (preservation number M207096). After strain activation, it was sent to the Beijing National Grain and Oil Research Institute for production and microencapsulation into a powder preparation (viable count $\geq 1.01\$\times 10\hat{}\{9\}\$$ CFU/g).

1.2 Experimental Diets

The experimental diets were formulated according to the nutrient requirements recommended by NRC (1998) for finishing pigs (60–110 kg). The composition and nutrient levels of the basal diet are presented in Table 1 . All diets were prepared as powder.

1.3 Experimental Design and Sample Collection

One hundred and twenty healthy "Duroc × (Landrace × Yorkshire)" finishing pigs with an average body weight of $(65.34\$\pm\$3.64)$ kg were randomly divided into two groups, with six replicates (pens) per group and ten pigs per replicate (half male and half female). The control group received the basal diet, while the experimental group received the basal diet supplemented with 0.10% *L. delbrueckii* preparation. The pre-trial period lasted 7 days, followed by a 42-day

trial period. At the end of the experiment, one pig with similar body weight was selected from each pen, fasted for 24 hours, and then blood was collected from the anterior vena cava before electrical stunning and slaughter. The ileum, liver, subcutaneous adipose tissue, and longissimus dorsi muscle were collected for sampling. Blood samples were allowed to stand for 30 minutes, then centrifuged at 3,000 r/min for 10–15 minutes to separate serum, which was aliquoted into 1.5 mL EP tubes and stored at -20°C. Tissue samples (5–10 g each) of ileum, liver, subcutaneous adipose tissue, and longissimus dorsi muscle were wrapped in aluminum foil and stored at -80°C. Subcutaneous adipose tissue samples (1 cm × 1 cm × 2 cm) were rinsed in physiological saline, then transferred to 10 mL EP tubes containing 10% formalin.

1.4 Feeding Management

The feeding trial was conducted at the Tianxin 502 Netling Fourth Supervision District Original Breed Pig Farm in Youxian County, Hunan Province, from May 31 to July 11, 2013. During the trial period, feed was provided twice daily at 09:00 and 16:00, with pigs allowed ad libitum access to feed and water. Other management and immunization procedures followed the farm' s standard protocols.

1.5 Analytical Methods

1.5.1 Serum Lipid Indexes Serum triglyceride (TG), total cholesterol (TC), glucose (GLU), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and total bile acid (TBA) concentrations were measured using a Mindray BS-200 automatic biochemical analyzer according to the instructions of the respective reagent kits.

1.5.2 Hepatic Enzyme Activities Related to Cholesterol Metabolism and Fat Deposition Approximately 0.5 g of liver sample was transferred to a 10 mL centrifuge tube, mixed with 5 mL phosphate-buffered saline (PBS) (pH=7.4), homogenized thoroughly, and centrifuged at 2,000–3,000 r/min for 20 minutes. The supernatant (400 μL) was collected and aliquoted into 1.5 mL EP tubes. Enzyme-linked immunosorbent assay (ELISA) kits from Shanghai Bogu Biological Technology Co., Ltd. were used to detect hepatic hydroxymethylglutaryl-CoA reductase (HMGR) and cholesterol 7α-hydroxylase (CYP7A1) activities. ELISA kits from Changsha Blange Biotechnology Co., Ltd. were used to measure hepatic lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), and adipose triglyceride lipase (ATGL) activities.

1.5.3 Tissue mRNA Expression of Genes Related to Cholesterol Metabolism and Fat Deposition Total RNA Extraction and Reverse Transcription: Ileum, liver, longissimus dorsi muscle, and subcutaneous adipose tissue samples were rapidly ground to a paste in liquid nitrogen-filled mortars. Approximately 20 mg of each sample was used for total RNA

extraction using the Trizol one-step method. Genomic DNA contamination was removed using gDNA Eraser. Total RNA concentration and purity were measured using a NANODROP 2000 spectrophotometer. RNA integrity was assessed by 1.0% agarose gel electrophoresis using 0.5 μg of total RNA. Reverse transcription was performed using a PrimeScript RT Master Mix kit according to the manufacturer' s instructions in a 20 μL reaction system. The synthesized cDNA was stored at -80°C.

Primer Design and Real-Time Quantitative PCR: Based on gene sequences published in GenBank (NCBI), primers for porcine β-actin, low-density lipoprotein receptor (LDLR), high-density lipoprotein receptor (HDLR), apical sodium-dependent bile acid transporter (ASBT), ileal bile acid binding protein (IBABP), peroxisome proliferator-activated receptor γ (PPARγ), and adipocyte differentiation determination factor 1 (ADD1) were designed using Primer Premier 5.0 software and synthesized by Shanghai Bioengineering Co., Ltd. (Table 2). Real-time quantitative PCR was performed using an ABI 7900HT system (ABI, USA) in a 20 μL reaction mixture containing 12.5 μL SYBR Premix Ex Taq II, 1.0 μL each of forward and reverse primers, 2 μL cDNA template, and 3.5 μL dH2O. The PCR conditions were: enzyme activation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. β-actin was used as the reference gene for data processing. The relative mRNA expression of target genes was calculated using the 2^(-ΔΔCt) method.

1.5.4 Histomorphological Observation of Subcutaneous Adipose Tissue Fixed subcutaneous adipose tissue samples were processed using paraffin sectioning techniques. Sections were observed and photographed using a Motic inverted biological microscope (400×). Six fields with intact adipose tissue morphology, clear outlines, and suitable for measurement were randomly selected from each section. Images were captured and analyzed using the Motic microscopic image analysis system to determine adipocyte number, diameter, and area.

1.6 Statistical Analysis

Experimental data were organized using Excel 2003 software and analyzed using SPSS 17.0 statistical software. Student's t-test was used to determine significant differences, with $P<0.05$ as the criterion for statistical significance. Results are expressed as mean (M) ± standard deviation (SD).

2. Results

2.1 Effects of *L. delbrueckii* on Serum Lipid Indexes in Finishing Pigs

As shown in Table 3 , serum TG, TC, GLU, and LDL-C concentrations in the experimental group decreased by 8.70%, 7.22%, 8.70%, and 6.30%, respectively, compared with the control group, while serum HDL-C concentration and HDL-C/LDL-C ratio increased by 2.70% and 10.34%, respectively. However, these

differences were not statistically significant (P>0.05). Serum TBA concentration was significantly reduced by 38.59% in the experimental group compared with the control group (P<0.05).

2.2 Effects of *L. delbrueckii* on Hepatic Enzyme Activities Related to Cholesterol Metabolism and Fat Deposition in Finishing Pigs

As shown in Table 4 , hepatic HMGR and LPL activities in the experimental group decreased by 10.80% and 3.31%, respectively, compared with the control group, but these differences were not significant (P>0.05). ATGL activity was significantly decreased by 22.37% in the experimental group (P<0.01). Compared with the control group, hepatic CYP7A1 and HSL activities in the experimental group increased by 25.79% (P<0.05) and 0.56% (P>0.05), respectively.

2.3 Effects of *L. delbrueckii* on Tissue mRNA Expression of Genes Related to Cholesterol Metabolism and Fat Deposition in Finishing Pigs

As shown in Table 5 , compared with the control group, the mRNA relative expression of hepatic LDLR and HDLR in the experimental group increased by 5.41% and 5.77%, respectively, but these differences were not significant (P>0.05). The mRNA relative expression of ileal ASBT and IBABP decreased by 3.52% and 11.11%, respectively, but these differences were also not significant (P>0.05). The mRNA relative expression of PPARγ and ADD1 in subcutaneous adipose tissue increased by 29.70% (P<0.05) and 13.33% (P>0.05), respectively. In the longissimus dorsi muscle, the mRNA relative expression of PPARγ and ADD1 increased by 9.62% and 24.32%, respectively, but these differences were not significant (P>0.05).

2.4 Effects of *L. delbrueckii* on Subcutaneous Adipose Tissue Morphology in Finishing Pigs

As shown in Table 6 , subcutaneous adipocyte number, diameter, and area in the experimental group decreased by 5.81%, 6.69%, and 4.76%, respectively, compared with the control group, but these differences were not statistically significant (P>0.05).

3. Discussion

3.1 Effects of *L. delbrueckii* on Serum Lipid Indexes in Finishing Pigs

Blood physiological indexes can be used to evaluate metabolic status and health in animals. Finishing pigs exhibit vigorous fat accumulation with relatively slow growth, and their capacity for body fat deposition increases with body weight [9-10]. Measurement of serum GLU and lipid indexes can partially reflect lipid metabolism and fat deposition capacity in finishing pigs. The present study showed that serum TG, TC, GLU, and LDL-C concentrations decreased,

while serum HDL-C concentration and HDL-C/LDL-C ratio increased in the experimental group, suggesting that *L. delbrueckii* plays a role in regulating lipid metabolism, improving blood lipid profiles, and reducing body fat deposition in finishing pigs. Serum TC, HDL-C, LDL-C, and TBA concentrations are important biochemical indicators of cholesterol metabolism. The decreased serum TC and LDL-C concentrations in the experimental group indicate that *L. delbrueckii* can reduce serum cholesterol levels in finishing pigs, which is consistent with numerous reports on cholesterol reduction by lactic acid bacteria [11-12]. TBA is the primary metabolite produced from cholesterol catabolism by CYP7A1 in the liver, and serum TBA concentration can reflect cholesterol utilization by the liver [10]. The significant reduction in serum TBA concentration in the experimental group suggests decreased cholesterol supply to the liver for bile acid synthesis, which aligns with the observed changes in serum TC concentration. Serum HDL-C concentration and HDL-C/LDL-C ratio reflect cholesterol clearance capacity. The increased serum HDL-C concentration and HDL-C/LDL-C ratio in the experimental group indicate improved efficiency of cholesterol transport to the liver and enhanced cholesterol clearance capacity, which is consistent with the reduced serum TC concentration. Previous studies have shown that the cholesterol-lowering mechanism of lactic acid bacteria is related to bile salt hydrolase (BSH) produced during colonization, growth, and reproduction in the intestine. BSH-active bacteria can consume bile salts synthesized by the liver and excreted into the intestine, increasing fecal bile acid excretion and consequently promoting cholesterol utilization for bile acid synthesis, thereby reducing serum cholesterol levels [13]. Our previous studies found that *L. delbrueckii* can promote fecal excretion of bile acids and cholesterol [7,10]. Therefore, the cholesterol-lowering mechanism of *L. delbrueckii* may be related to BSH-promoted bile acid excretion.

3.2 Effects of *L. delbrueckii* on Hepatic Enzyme Activities Related to Cholesterol Metabolism and Fat Deposition in Finishing Pigs

The liver is the primary site for cholesterol and bile acid synthesis. HMGR and CYP7A1 are the key enzymes in cholesterol and bile acid synthesis, respectively. Dietary regulation can interfere with intestinal absorption of cholesterol and bile acids, increase fecal excretion, and modulate hepatic HMGR and CYP7A1 activities and mRNA expression [14-17]. Li et al. [10] reported that *L. delbrueckii* can promote fecal excretion of bile acids and cholesterol. In this study, hepatic HMGR activity decreased while CYP7A1 activity significantly increased in the experimental group compared with the control group. Decreased HMGR activity reduces endogenous cholesterol synthesis in the liver, leading to reduced serum TC concentration and consequently decreased LDL-C concentration. Increased CYP7A1 activity enhances bile acid synthesis and cholesterol catabolism, improving cholesterol transport efficiency to the liver and increasing serum HDL-C concentration [18]. Similar results were observed in the serum lipid indexes measured in this study.

LPL, HSL, and ATGL play important roles in TG synthesis and degradation in adipocytes. LPL is the key enzyme for adipocyte uptake of free fatty acids (FFA), catalyzing the hydrolysis of TG carried by chylomicrons (CM) and very low-density lipoprotein (VLDL) in blood to glycerol and FFA, which enter cells via the CD36 pathway [19]. HSL and ATGL are rate-limiting enzymes for intracellular TG hydrolysis [20]. In this study, decreased hepatic LPL and ATGL activities in the experimental group suggest inhibition of TG synthesis pathways and reduced lipid synthesis in hepatic adipocytes, while also indicating suppressed TG degradation and reduced release of glycerol and FFA. These findings suggest that *L. delbrueckii* may have a protective effect against fatty liver disease.

3.3 Effects of *L. delbrueckii* on Tissue mRNA Expression of Genes Related to Cholesterol Metabolism and Fat Deposition and Subcutaneous Adipose Tissue Morphology

Cholesterol in blood is transported primarily by LDL-C and HDL-C. LDLR is a single-chain transmembrane glycoprotein receptor present on nearly all cell membranes, mediating the uptake of cholesterol ester-rich VLDL-C and LDL-C from plasma into tissues for metabolism and clearing excess lipids (particularly cholesterol) from blood vessels. Since LDL in blood is derived from VLDL, LDL-C accounts for two-thirds of total cholesterol transport [21-22]. Insufficient or absent cell surface LDLR reduces LDL-C uptake from blood, decreasing cellular cholesterol utilization and causing cholesterol deposition in blood vessels, which can lead to cardiovascular disease [23]. Excess cholesterol can also be transported to the liver as HDL-C for metabolism through a process called reverse cholesterol transport (RCT). HDL acts as a scavenger, carrying blood cholesterol to hepatocytes via HDLR binding for metabolic conversion to bile acids. The number of hepatocyte surface HDLRs partially determines the amount of serum cholesterol entering hepatic metabolism [24-25]. In this study, increased mRNA expression of hepatic LDLR and HDLR in the experimental group indicates enhanced hepatic cholesterol metabolism, providing a basis for the observed reduction in serum TC concentration.

Intestinal bile acid reabsorption is a critical component of enterohepatic circulation, occurring primarily in the ileum [26]. As bile acids flow through the ileum, apical sodium-dependent bile acid transporter (ASBT) mediates bile acid transport, which then binds to IBABP and is reabsorbed into blood via the basolateral terminal sodium-dependent bile acid transporter (tASBT) for return to the liver via the portal vein. Under normal conditions, 95% of bile acids can be reabsorbed via ASBT-mediated transport. Studies have shown that interrupting enterohepatic bile acid circulation can enhance hepatic cholesterol metabolism and reduce serum LDL-C concentration. Additionally, ASBT expression is related to intestinal bile acid pool size, fecal sterol excretion, and CYP7A1 activity [27-29]. In this study, decreased mRNA expression of ileal ASBT and IBABP in the experimental group suggests impaired ileal bile acid

reabsorption, potentially altering enterohepatic bile acid circulation. Previous studies have shown that feeding *L. delbrueckii* enhances hepatic CYP7A1 activity and increases fecal bile acid excretion [7,10]. Therefore, we can infer that the cholesterol-lowering mechanism of *L. delbrueckii* in finishing pigs may be related to its interference with ileal bile acid circulation, which is consistent with the speculation by De Rodas et al. [30], although the specific interference process requires further investigation.

Intramuscular and subcutaneous fat are important indicators for pork quality evaluation. Fat deposition in tissues is regulated by a series of transcription factors related to adipocyte differentiation. ADD1 is an important nuclear transcription factor in animal lipid metabolism that regulates lipid synthesis by modulating the expression of genes encoding lipid metabolism-related enzymes [31]. PPARγ is a key factor regulating adipocyte differentiation. Upon activation, it can regulate the expression of lipid metabolism target genes, and many genes related to fatty acid transport and metabolism are transcriptionally regulated by PPARγ [32]. Studies have shown that increased PPARγ gene expression in adipose tissue is often accompanied by decreased blood glucose and lipid concentrations [33-34], which is consistent with our results. Therefore, *L. delbrueckii* appears to influence the expression and activity of adipocyte differentiation transcription factors in tissues, thereby exerting regulatory effects on body fat deposition. Additionally, the histomorphological results of subcutaneous adipose tissue in this study showed that adipocyte number, diameter, and area in the experimental group were numerically lower than those in the control group, further reflecting the regulatory effect of *L. delbrueckii* on body fat deposition in finishing pigs.

In conclusion, dietary supplementation with 0.10% *L. delbrueckii* can affect ileal bile acid reabsorption, interfere with enterohepatic bile acid circulation, and regulate hepatic cholesterol and lipid metabolism in finishing pigs.

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Note: Figure translations are in progress. See original paper for figures.