Combined effect of *Lactobacillus acidophilus* and β-cyclodextrin on serum cholesterol in pigs

L. Alonso[1,2]*, J. Fontecha[3] and P. Cuesta[2]

[1]*Instituto de Productos Lácteos de Asturias (CSIC), Paseo Rio Linares s/n 33300, Villaviciosa, Asturias, Spain*
[2]*Department of Animal Science, Food Agricultural Products Center, Oklahoma State University, Stillwater, OK 74075, USA*
[3]*Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Universidad Autónoma de Madrid, 28049, Madrid, Spain*

Abstract

A total of twenty-four Yorkshire gilt pigs of 6–7 weeks of age were used in a 2×2 factorial experiment to determine the individual and combined effects of the inclusion of two dietary factors (cholesterol rich, 3 % β-cyclodextrin (BCD) and *Lactobacillus acidophilus* cultures) on total cholesterol and LDL-cholesterol levels in blood serum. Pigs were assigned randomly to treatment groups (n 6). Total serum cholesterol concentrations decreased after 3 weeks in all the experimental treatment groups, including diets with BCD, *L. acidophilus* or both. Similar trends were observed for serum LDL-cholesterol concentrations among the experimental treatments. No statistically significant differences from the control group were observed in either total serum cholesterol or LDL-cholesterol concentrations ($P < 0.05$) for each of the individual treatment groups: BCD or *L. acidophilus*. However, significant differences in total serum cholesterol concentrations were observed when comparing the combined treatment group (BCD and *L. acidophilus*) with the control group, which consisted of a basal diet and sterile milk. The combined treatment group exhibited 17·9 % lower total serum cholesterol concentration after 3 weeks. Similar significant differences were observed when comparing the combined effect experimental group with the control group after 3 weeks. The combined treatment group exhibited 27·9 % lower serum LDL-cholesterol concentrations.

Key words: β-Cyclodextrin: *Lactobacillus acidophilus*: Cholesterol: Pigs

Heart disease is a major cause of death in humans[1]. Nutritional studies have indicated that high concentrations of total cholesterol (TC) and LDL-cholesterol correlate highly with the incidence of CHD. Thus, considerable research has been conducted to determine factors that are effective in lowering concentrations of serum cholesterol, including dietary modifications[2]. β-Cyclodextrin (BCD) is a cyclic oligosaccharide consisting of seven glucose units and is produced from starch using the enzyme cyclodextrin glycotransferase – to break the polysaccharide chain – giving the molecule its affinity for molecules such as cholesterol[3–5]. Several reports[6,7] have indicated that consumption of certain cultured dairy products supplemented with *Lactobacillus acidophilus* reduced concentrations of serum cholesterol during growth in laboratory media. The presence of bile and anaerobic conditions during growth enable *L. acidophilus* to assimilate cholesterol[8–10]. Such assimilation of cholesterol in the small intestine may be important for reducing the absorption of dietary cholesterol from the digestive system into the blood. Pigs were selected as the animal model for this study as their digestive system, distribution of coronary arteries and atherosclerotic tendencies resemble those of humans[11].

As *L. acidophilus* exhibits host specificity in the intestinal tract[12], we had to use strains of *L. acidophilus* of pig origin.

The objective of the present study was to investigate the effects of a BCD diet rich in cholesterol supplemented with a culture of *L. acidophilus* on TC and LDL-cholesterol levels in blood serum in twenty-four 6–7-week-old Yorkshire gilts randomly assigned to individual pens, divided into four treatments, with six pigs per treatment.

Methods

Chemicals

BCD was obtained from Cavamax w7 (Wacker) and cholesterol (Sigma Co.). All the other reagents were of the highest commercially available quality.

Source of culture and bile tolerance

L. acidophilus EP32 used in this experiment was from our laboratory stock culture collection and was originally isolated from the intestinal contents of pigs[8]. The organisms were

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cultured in the presence of bile by individual inoculation (1 %) into sterile MRS-THIO broth (lactobacilli de Man, Rogosa and Sharpe broth supplemented with 0·2 % sodium thioglycolate) with and without 0·3 % oxgall (bovine bile), and the culture was incubated for 3 h at 37°C. Increases in absorbance at 620 nm during the 2-h incubation period were used to compare growth of the culture.

Deconjugation of sodium taurocholate and β-cyclodextrin

About 30 ml of MRS-THIO broth containing 0·2 % sodium taurocholate was inoculated (1 %) with *L. acidophilus* EP32 and incubated at 37°C in the presence of 1 % BCD. One tube of each culture was removed at 3-h intervals throughout the 18 h of incubation. A 1:10 dilution was made from each tube using sterile peptone diluent (1 %), and the absorbance at 620 nm was measured to determine the relative amount of growth. Free cholic acid liberated by each culture was measured[13]. Absorbance was read at 660 nm against a reagent blank and compared with a standard curve to determine the concentration of free cholic acid. The results are expressed as micromoles of cholic acid per millilitre.

Animals

A total of twenty-four 6–7-week-old Yorkshire gilt female pigs weighing approximately 9–10 kg obtained from the pig research unit, Oklahoma State University, were used for the study. All the procedures involving animals in this study were conducted with the approval of the Oklahoma State University, Animal Ethics Committee.

Experimental design

Pigs were randomly assigned to individual pens and subsequently divided into four treatment groups with six pigs per treatment. The location of each pig was random with respect to the treatments (Fig. 1). The experimental basal diet (902 kg) was prepared by mixing using a Marion mixer (Rapids Machinery Co.); 228·0 kg of the diet was removed for adjustment period feeding, and 2·1 kg of cholesterol, with purity at least equivalent to the United State Pharmacopeia recommendations (Sigma Co.),

was added and mixed to the remaining 674·0 kg. Including cholesterol contents from butter, the experimental diet contained 1775 mg of cholesterol/kg. After mixing, 10·0 kg BCD was added to 337·0 kg to provide the BCD experimental diet. All the pigs were fed a maize basal diet for the adjustment period for 1 week without added crystalline cholesterol twice daily (morning and afternoon); the experimental period began at the 2nd week. All the pigs were then fed the maize experimental diet (Table 1) at the start of the trial according to the four treatment groups: 1M, control high-cholesterol diet plus 50 ml of sterile non-fat milk; 1C, high-cholesterol diet plus 50 ml of sterile non-fat milk containing 5×10^{10} cells of *L. acidophilus* EP32; 2M, 3 % BCD high-cholesterol diet plus 50 ml of sterile non-fat milk; and 2C, 3 % BCD high-cholesterol diet plus 50 ml of sterile non-fat milk containing 5×10^{10} cells of *L. acidophilus* EP32. Pigs were fed

Table 1. Ingredients and nutritional values of the experimental basal diets

	1M	1C	2M	2C
Ingredients (g/kg)				
Ground shelled maize	417·3	417·3	405·1	405·1
Butter	80·4	80·4	78·0	78·0
Dried sweet whey	198·6	198·6	192·9	192·9
Soyabean meal	269·4	269·4	261·3	261·3
Salt	2·2	2·2	2·1	2·1
Dicalcium phosphate	15·5	15·5	15·0	15·0
Calcium carbonate	8·9	8·9	8·1	8·1
Vitamin and trace mineral premix*	5·9	5·9	5·7	5·7
β-Cyclodextrin	–	–	30·0	30·0
Cholesterol	1·8	1·8	1·8	1·8
Nutritional value (g/100 g)				
L-Lysine	0·8	0·8	0·7	0·7
Protein	16·1	16·1	12·4	12·4
Total fat	12·8	12·8	9·8	9·8
Carbohydrate	49·5	49·5	38·1	38·1
Energy (kJ/100 g)	1930·1	1930·1	1684·7	1684·7

1M, control high-cholesterol diet plus 50 ml of sterile non-fat milk; 1C, high-cholesterol diet plus 50 ml of sterile non-fat milk containing 5×10^{10} cells of *Lactobacillus acidophilus* EP32; 2M, 3 % β-cyclodextrin (BCD) high-cholesterol diet plus 50 ml of sterile non-fat milk; 2C, 3 % BCD high-cholesterol diet plus 50 ml of sterile non-fat milk containing 5×10^{10} cells of *L. acidophilus* EP32.
* The vitamin trace mineral premix supplied 1760 mg of riboflavin, 8800 mg of pantothenic acid, 8800 mg of niacin, 8·8 mg of vitamin B_{12}, 176 000 mg of choline chloride, 528·28 mg of vitamin A, 4·42 mg of vitamin D_3, 3235·29 mg of vitamin E, 44 mg of menadiane dimethyl-primidional bisulfite, 39·6 mg of Se, 299·2 mg of I, 19·8 g of Fe, 11 g of Mn, 2·2 g of Cu and 39·6 g of Zn/kg of premix.
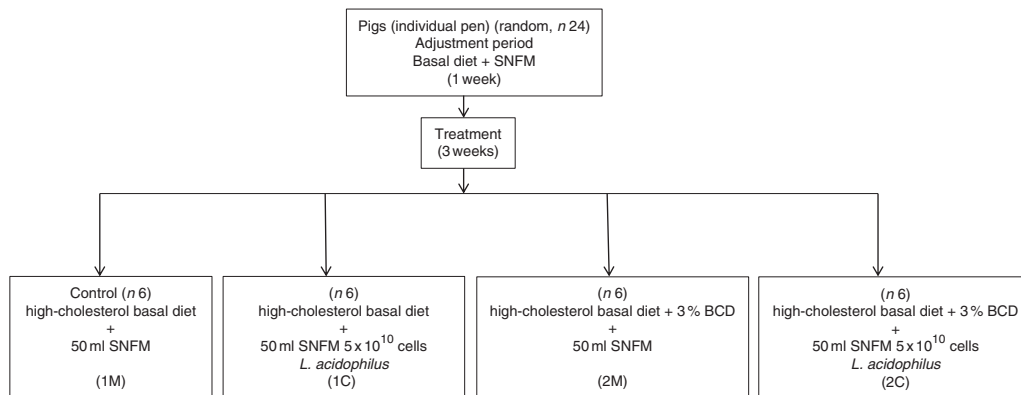

Fig. 1. Flow chart detailing the experimental design of each treatment. SNFM, sterile non-fat milk; BCD, β-cyclodextrin; *L. acidophilus*, *Lactobacillus acidophilus*.

twice daily and water was available at all times. During the experimental period, the pigs were fed for 21 d at 10 % of the metabolic body weight ($kg^{0.70}$). If food was refused following the morning meal, it was offered again with the afternoon meal. The quantity of food remaining at the end of the day was recorded.

Blood collection

Blood samples were collected weekly from the indwelling jugular catheter just before the 1st day of the training period and continued for additional 28 d after initiation of the trial. Immediately following collection, the tubes were placed in ice-cold water bags and then centrifuged for 20 min at 3000 **g**, and the serum samples were transferred into screw-cap vials and stored at −20°C until the samples were analysed. Duplicate samples of serum were analysed for TC. Concentrations of LDL-cholesterol were calculated by the difference between TC and HDL-cholesterol. All the analyses were performed using enzymatic kits (Sigma Chemical Co.).

Statistical analysis

The statistical design was a 2 × 2 factorial design, and time was included as the factor for repeated-measures analysis to determine the significance of meal effect (treatment 1 (cyclodextrin: yes, no), treatment 2 (probiotic: yes, no), time) and interactions (treatment 1 × treatment 2, treatment 1 × time, treatment 2 × time, treatment 1 × treatment 2 × time). For each time point ANOVA (two-way full factorial) was performed using SAS software[14]. Statistical significance was accepted at $P < 0.05$.

Results

All the animals remained in good health throughout the experiment. There were no significant differences ($P < 0.05$) in feed intake and weight gain as well as the growth and development between treatments.

The effects of *Lactobacillus* and BCD on serum TC and LDL-cholesterol during the 4 weeks of treatment are presented in Fig. 2, according to the statistical model presented in experimental methods. Pigs fed *L. acidophilus* and BCD (2C) had a significant interaction ($P = 0.021$) of plasma TC than those without BCD and *L. acidophilus* (1M) (2·63 (SD 0·21) *v.* 3·20 (SD 0·26) mmol/l) (17 % lower) at the end of the treatment period. Similarly, pigs fed BCD and milk (2M) had a significant interaction ($P = 0.028$) of TC than those without BCD and *L. acidophilus* (2C) (2·82 (SD 0·27) *v.* 3·20 (SD 0·26) mmol/l) with a decrease of 12 %. No significant interaction ($P = 0.253$) was observed between basal diet with *L. acidophilus* and BCD (1C, 2M) in the two experimental groups at 3 weeks (2·93 (SD 0·24) *v.* 2·82 (SD 0·27) mmol/l). As expected, total serum cholesterol concentrations decreased for all the treatment groups containing *L. acidophilus*, BCD or both in the diet after 3 weeks of treatment. Similar results were observed for LDL-cholesterol for the four treatments. No significant interactions ($P = 0.089; 0.095; 0.091$) were observed for the separate treatments with *L. acidophilus* and BCD diets (1C, 2C and 2M) (1·42 (SD 0·18), 1·28 (SD 0·18) and 1·25 (SD 0·22) mmol/l), but a significant interaction ($P = 0.022; 0.029; 0.023$) of those treatments with the control group was found (basal diet and milk, 1M) (1·83 (SD 0·18) mmol/l) with a decrease of 23 %. The main significant interaction ($P = 0.017$) was for the group fed *L. acidophilus* and BCD (2C) compared with the control (1M) (1·28 (SD 0·18) *v.* 1·83 (SD 0·18) mmol/l) with a decrease of over 27 % after 3 weeks of treatment.

Discussion

Serum TC concentrations increased, as expected, by a diet supplemented with crystalline cholesterol and butter. This ability to increase serum cholesterol levels of pigs by increasing dietary cholesterol has been reported in other studies[15,16]. High concentrations of serum TC and LDL-cholesterol are strongly associated with an increased risk for CHD[17]. Reduction in TC and LDL-cholesterol in hypercholesterolomic
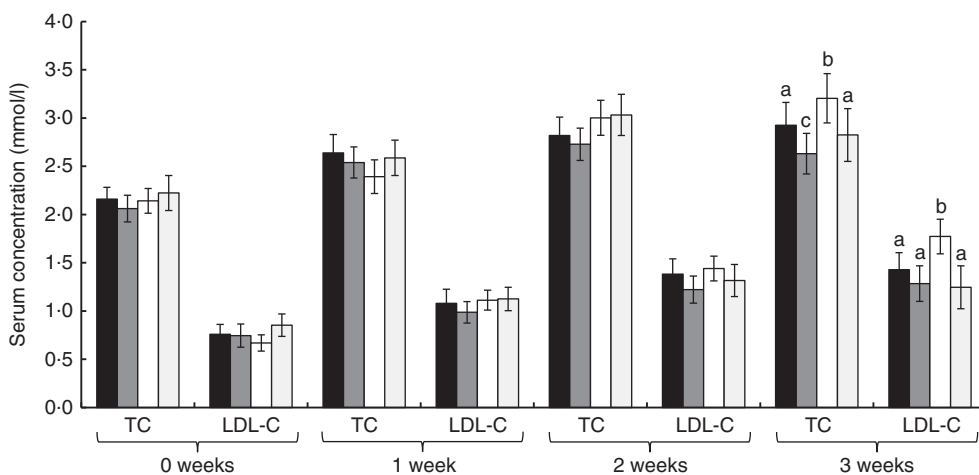

Fig. 2. Serum concentrations (mmol/l) of total cholesterol (TC) and LDL-cholesterol of pigs previously fed a high-cholesterol diet with β-cyclodextrin (BCD) and *Lactobacillus acidophilus* during the three experimental weeks. Treatments sharing the same letter are not significantly different at 95 % CI. ☐, Milk; ■, milk + *L. acidophilus*; ☐, milk + BCD; ■, *L. acidophilus* + BCD.

men has been reported to reduce the incidence of CVD[14]. Thus, to assay different ways to reduce serum cholesterol levels are important. This study shows that dietary supplementation with *L. acidophilus* EP32 and BCD diet reduced total serum cholesterol in pigs previously fed a high-cholesterol diet more significantly compared with pigs that did not receive *L. acidophilus* and BCD. These findings are in accordance with those reported for *L. acidophilus* in humans[18], swine[8] and rats[19] as well as for BCD in pigs[20]. At present, there are no studies that have combined *L. acidophilus* and BCD in the same diet. Some authors, using other strains of *Lactobacillus*, have found no effect of *L. acidophilus* on serum cholesterol concentration[21], and a possible explanation for these results is that the strain of *L. acidophilus* used in the study was not selected for its ability to take up cholesterol during growth or its ability to deconjugate bile acids. In fact, the *L. acidophilus* assayed by those authors was only moderately active in assimilating cholesterol and bile resistance[22], and no data have been reported on its ability to deconjugate bile acids. These factors may have limited the ability of this strain to survive and grow in the intestinal tract and to exert a beneficial effect by regulating serum cholesterol levels. The *L. acidophilus* EP32 used in our study is very active in taking up cholesterol and in deconjugating bile acids[13]. *L. acidophilus* EP32 tested in this study in the presence of BCD exhibited a greater degree of bile tolerance and the ability to deconjugate sodium taurocholate releasing more cholic acid and growing slightly more in the broth media with BCD. Deconjugation of sodium taurocholate in the *in vitro* assay in this study was higher ($P < 0.05$) when *L. acidophilus* was grown in presence of 1% BCD (4.4 ± 0.4 mM) compared with controls (3.1 ± 0.3 mM) as BCD enhances deconjugation of bile salts.

Deconjugated bile acids are less well absorbed from the small intestine than are the conjugated bile acids. Thus, deconjugation of bile acids in the small intestine could result in greater excretion of bile acid from the intestinal tract, especially because free bile acids are excreted more rapidly than conjugated bile acids[9,10]. An excretion of bile acids should result in lower serum bile acids, which in turn would decrease the amount of bile acids reaching the liver for secretion back into the intestine via the enterohepatic circulation[23].

Taking into account the high capacity of BCD to bind to cholesterol[24] it may be assumed that BCD acts as a cholesterol trap in the lumen, and thus inhibits the absorption of both dietary and endogenous cholesterol in the small intestine. However, endogenous cholesterol entering the lumen from the bile can act as a competitor for the binding process[25]. The inhibition of cholesterol absorption is not sufficient to explain the overall action of BCD with respect to bile acid metabolism. As BCD binds bile acids with a relatively high affinity *in vitro*[20] and enhances the deconjugation of bile salts by *L. acidophilus* EP32 in bile acids in the combined diet of this study, it is likely that this carbohydrate also plays a role analogous to that described for bile acid chelators such as cholestyramine[26]. These compounds, which are not metabolised in the digestive tract, may act as hypocholesterolaemic agents in pigs by these mechanisms. In conclusion, addition of BCD to the cholesterol-rich diet plus *L. acidophilus* prevented the elevation of plasma LDL-cholesterol and lowered TC levels.

References

1. Atkins JL, Whincup PH, Morris RW, et al. (2014) High diet quality is associated with a lower risk of cardiovascular disease and all cause mortality in older men. *J Nutr* **144**, 673–680.
2. Kannel WB, Doyle JT, Osteld AM, et al. (1984) Optimal resources for primary prevention of atherosclerotic diseases. *Circulation* **70**, 157–165.
3. Alonso L, Cuesta P, Fontecha J, et al. (2009) Use of beta-cyclodextrin to lower level of cholesterol in milk fat. *J Dairy Sci* **3**, 863–869.
4. Reineccius TA, Reineccius GA & Peppard TL (2004) Utilization of beta-cyclodextrin for improved flavor retention in thermally processed foods. *J Food Sci* **69**, 58–62.
5. Szente L & Szejtli J (2004) Cyclodextrins as food ingredients. *Trends Food Sci Technol* **15**, 137–142.
6. DiRienzo DB (2014) Effect of probiotics on biomarkers of cardiovascular disease: implications for heart-healthy diets. *Nutr Rev* **72**, 18–19.
7. Stancu CS & Deleanu GM (2014) Probiotics determine hypolipidemic and antioxidant effects in hyperlipidemic hamsters. *Mol Nutr Food Res* **56**, 559–568.
8. Gilliland SE, Nelson CR & Maxwell C (1985) Assimilation of cholesterol by *Lactobacillus acidophilus*. *Appl Environ Microbiol* **49**, 377–383.
9. Noh DO, Kim SH & Gilliland SE (1997) Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. *J Dairy Sci* **80**, 3107–3113.
10. Corzo G & Gilliland SE (1999) Measure of bile salt hydrolase activity from *Lactobacillus acidophilus* base on disappearance of conjugated linoleic bile salts. *J Dairy Sci* **82**, 466–471.
11. Ratcliffe HL (1971) The domestic pig: a model for experimental atherosclerosis. *Atherosclerosis* **13**, 133–136.
12. Gilliland SE, Bruce BB & Bush J (1980) Comparison of two strains of *Lactobacillus acidophilus* as dietary adjuncts for young calves. *J Dairy Sci* **63**, 964–972.
13. Walker DK & Gilliland SE (1993) Relationships among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*. *J Dairy Sci* **76**, 956–961.
14. SAS Institute (2006) *User's Guide. Statistics, Version 9.1.3.* Cary, NC: SAS Institute Inc.
15. Jensen TW, Mazur MJ, Pettigew JE, et al. (2010) A cloned pig model for examining atherosclerosis induced by high fat, high cholesterol diets. *Anim Biotechnol* **3**, 179–187.
16. Sun J, Monagas M, Jang S, et al. (2015) A high fat, high cholesterol diet leads to changes in metabolite patterns in pigs – a metabolomic study. *Food Chem* **173**, 171–178.

17. Lipid Research Clinics Program (1984) The lipid research clinics coronary primary prevention trial results. I. Reduction in incidence of coronary heart disease to cholesterol lowering. *JAMA* **251**, 351–369.

18. Harrison VC & Peat G (1975) Serum cholesterol and bowel flora in the newborn. *Am J Clin Nutr* **28**, 1351–1359.

19. Grunewald KK (1982) Serum cholesterol levels in rats fed skim milk fermented by *Lactobacillus acidophilus*. *J Food Sci* **47**, 2078–2086.

20. Juste C, Domingo N, Lafont H, et al. (1997) Inducing cholesterol precipitation from pig with beta-cyclodextrin and cholesterol dietary supplementation. *J Hepatol* **26**, 711–721.

21. Lin SY, Ayres JW, Winkler JR, et al. (1989) Lactobacillus effects on cholesterol: *in vitro* and *in vivo* results. *J Dairy Sci* **72**, 2885–2895.

22. Gilliland SE & Walker DW (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J Dairy Sci* **73**, 905–914.

23. Pereira DI & Gibson GR (2002) Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Crit Rev Biochem Mol Biol* **37**, 259–281.

24. Riottot M, Oliver P, Caboche M, et al. (1993) Hypolipidemic effects of beta-cyclodextrin in the hamster and in the genetically hypercholesterolemic Rico rat. *Lipids* **28**, 181–188.

25. van Munster IP, Tangerman A & Nagengast M (1994) Effect of resistant starch on colonic fermentation, bile acid metabolism, and mucosal proliferation. *Dig Dis Sci* **39**, 834–842.

26. Boucarel B, Ceryak S, Robins SJ, et al. (1995) Studies on the mechanism of the ursodeoxycholic acid-induced increase in hepatic low-density lipoprotein binding. *Lipids* **30**, 607–617.