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Assimilation of cholesterol in broth, cream, and butter by probiotic bacteria

High serum cholesterol concentrations are associated with the development of coronary heart disease. It has been reported that some cultures of *Lactobacillus* spp. actively take up cholesterol from laboratory media. In the present study, the abilities of ten probiotic lactic acid bacteria to assimilate cholesterol in broth medium, cream, and butter were tested and compared. The cholesterol reduction ratios of these human-origin bacteria were determined in MRS-THIO broth supplemented with 150 μg/mL cholesterol. The amount of cholesterol assimilated by the bacteria was measured by gas chromatography. Cholesterol assimilation of these ten bacteria in broth was found to be similar to assimilation in cream. Two of these ten bacteria (*Lactobacillus maltaramicus* AC 3–16 and *L. casei* subsp. *casei* AB16–65) were chosen for making soured butter. The results indicate that the probiotic bacteria applied to cream and butter, as well as to the broth medium, caused a reduction of the cholesterol level of the product's fat content. This study provides some evidence that probiotic cultures have a cholesterol level-reducing effect, and soon we will be able to produce butter without cholesterol *via* microorganisms.

Keywords: Cholesterol assimilation, probiotic bacteria, butter.

1 Introduction

Cholesterol is a natural component of milk fat and appears in milk of all animal species. It is an organic material of cell membranes and important for human metabolism. Several studies have indicated that the cholesterol levels in the blood and the consumption of diets rich in cholesterol are closely related to each other. This resulted in a trend to consume food with less cholesterol content. An increased supply of cholesterol can lead to hypercholesterolemia and it can accelerate the formation of atherosclerotic changes in the circulatory system [1–5]. Several researchers have shown that cardiovascular disease is a leading cause of death, similar to cancer. The studies of the trends of the causes of coronary hearth disease indicate that a high concentration of cholesterol in the blood for long periods is a risk factor of this disease. The relationship between the concentration of cholesterol and coronary hearth disease has prompted the medical profession to advise their patients to reduce their blood cholesterol levels [6]. Lactic acid bacteria are termed probiotic and are used as health adjuncts in food to provide a wide variety of health benefits. These bacteria, which are mainly lactobacilli and bifidobacteria, may have several therapeutic functions, including antimicrobial activity, anticholesterol activity, improved lactose utilization and anticarcinogenic activity [7–9]. For this reason, there has been considerable interest in the beneficial effects of fermented milk products containing probiotic bacteria on the human lipid metabolism. Several human studies have suggested a moderate cholesterol-lowering action of dairy products that have been fermented with certain strains of probiotic bacteria [10–13].

There are many workers in this field who use chemical application of β-cyclodextrin to reduce cholesterol [14–17]. However, because of the high cost of β-cylodextrin, this results in a relatively expensive product. Where physical methods are used for the same purpose, a loss of aroma of the product is experienced and, as a result, these methods are not always preferred [6]. On the other hand, biological methods of reducing cholesterol do not have any additional expense and it is thought that they would have favorable effects on aroma, taste and texture. However, there are very few published results with regard to reducing cholesterol in food products by way of microorganism assimilation.

The objective of this study was to test and compare the abilities of ten probiotic lactic acid bacteria to assimilate cholesterol in broth, cream, and butter. We hope this study provides some evidence that probiotic cultures have a cholesterol level-reducing effect, and that soon we will be able to produce butter without cholesterol *via* microorganisms.

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2 Materials and methods

2.1 Source and maintenance of cultures

The bacteria were isolated previously from feces samples of 13 healthy persons and from gastric biopsy samples of 21 persons who have gastride or non-specific gastride. Ninety-six *Lactobacillus* and 33 *Enterococcus* strains were identified and probiotic characteristics were tested [18]. Stock cultures were maintained at −80 °C in Crossley Milk Medium (Oxoid) with paraphine. Working cultures were maintained and subcultured in MRS-THIO Broth (MRS Broth; Merck) supplemented with 0.2% (wt/vol) sodium thioglycolate (Sigma) and 0.3% (wt/vol) oxgall (Sigma).

2.2 Assimilation of cholesterol

The amounts of cholesterol removed by the test cultures and those in the control broth which had not been inoculated were determined by gas chromotography (GC) methods described in Fleutouris *et al*. [19]. The test medium used for screening cultures for cholesterol uptake was sterile modified MRS broth supplemented with 0.2% (wt/vol) sodium thioglycolate, 0.3% (wt/vol) oxgall, and 150 mg/L of a water-soluble form of cholesterol (Sigma). The cultures were fermented for 24 h at 37 °C anaerobically by using GasPak Anaerobic System (Oxoid). After incubation, bacterial cells were removed by centrifugation (12000 × g, 10 min, 1 °C) and the spent broth and uninoculated control broths were then assayed for their cholesterol content. Strains were compared for cholesterol assimilation in terms of their specific cholesterol uptake after the 24-h incubation period.

2.3 Reduction of cholesterol content in cream and butter with lactic acid bacteria cultures

2.3.1 Preparation of cultures

Overnight cultures of strains were inoculated (2%) into cream containing 15% fat, and the mixtures were incubated anaerobically at 37 °C for 24 h. At the end of the incubation period, the cultures were centrifuged for 10 min at 12000 × g and the amount of cholesterol that had been removed by the culture was determined by GC methods described in Fleutouris *et al*. [19]. The control samples were prepared under the same conditions but were not inoculated with the bacteria.

The cultures for butter were prepared in the same way as for the cream, but in this case the cream's fat content was 35%. The sour cream that formed after 24 h of incubation was used for the production of butter [20]. In this instance, the same procedure that was followed in the case of cream was followed in determining the quantity of cholesterol.

2.3.2 Preparation of cholesterol standards

The stock solution (2 mg/mL) was prepared by dissolving 20 mg reference standard (Sigma) with hexane in a 10-mL volumetric flask. Working solutions were prepared by appropriately diluting the stock solution with hexane to obtain aliquots in the range of 10–80 μg/mL.

2.3.3 GC conditions

A fused-silica capillary column (Phenomenex Zebron ZB1 15 m × 0.32 mm i.d. × 1.0 μm df) was used. The oven temperature was set at 285 °C isothermal, the injection port temperature at 300 °C and the detector temperature at 300 °C. Liquid phase: 100% methyl polysiloxane. The flow rates were 2 mL/min for helium, 40 mL/min for hydrogen, and 400 mL/min for air. The injection volume was 1 μL.

2.4 Statistical analysis

Data from the replications of the experiments were analyzed using the ANOVA procedure from SAS (SAS 0.7.1998).

3 Results and discussion

In this study, 96 *Lactobacillus* and 33 *Enterococcus* strains were chosen to examine their ability to reduce cholesterol, and ten probiotic and lactic acid bacteria selected from these were tested to reduce cholesterol in broth and cream.

The cholesterol assimilation of these strains from MRS-THIO broth containing sodium taurocholate, oxgall and cholesterol during 24 h of anaerobic growth at 37 °C was determined (Tab. 1). Uninoculated sterile broth was also analyzed, as control. The cholesterol assimilation ranged from 12.10 to 47.54%. Among the strains tested, *L. agilis* BK 10–47 achieved the highest cholesterol removal (Tab. 1).

The results indicated that each culture behaved differently in the assimilation of cholesterol from the broth ($p < 0.01$). The amount of cholesterol assimilated was strain specific [21]. It was seen that assimilation of cholesterol increased when the pH values were lower (Tab. 1). The effect of the

Tab. 1. Assimilation of cholesterol (%) in MRS-THIO broth, pH degree and plate counts in MRS broth.

Lactic acid bacteria	Cholesterol assimilation [%]	pH	Plate counts[§]
L. casei subsp. *casei* BK10–48	24.33	5.29	8.00
L. intestinalis AC18–85	22.86	5.26	8.20
L. agilis BK10–47	47.54	4.49	6.74
L. agilis AB7–35	35.30	4.57	9.18
E. faecalis BC21–104	12.10	5.35	8.18
L. casei subsp. *casei* AB16–65	38.98	5.10	7.35
L. maltaromicus A21–101	40.60	4.62	7.70
L. maltaromicus AC3–16	36.77	4.57	7.70
L. agilis AC3–10	36.83	4.83	8.78
E. malodaratus BK9–43	41.90	4.57	6.18

[§] Reported as $\log_{10}$ colony-forming units per milliliter.

pH is an important parameter for the assimilation of cholesterol ($p < 0.01$). Brashears *et al*. [21] revealed that the amount of cholesterol that was removed from the broth was variable, depending on the culture and the pH, during growth.

The amount of cholesterol assimilated and the pH degrees in sour cream (15% fat content) together with the control sample are shown in Tab. 2. All strains examined were able to assimilate cholesterol to some extent. The amount of cholesterol assimilated by the cultures ranged from 59.78 to 20.62% in cream. The obtained results of this study confirm the ability of microorganisms to reduce cholesterol during growth in cream and butter.

The range of this reduction depends on the properties of the selected probiotic bacteria. *L. maltaramicus* AC 3–16, which has the highest cholesterol assimilation ability, and *L. casei* subsp. *casei* AB16–65, which is widely used in fermented dairy products, were selected for butter production. *L. casei* is slowly acidifying and forms L(+)-lactic acid as well as flavor formers, such as diacetyl [22]. While control butter was produced without cultures, the soured butter was produced using both cultures. The quantities of cholesterol in butter, after the incubation, the pH and the ratio of cholesterol assimilation are shown in Tab. 3.

Tab. 2. Quantity of cholesterol, pH degree and cholesterol assimilation in cream.

Lactic acid bacteria	Quantity of cholesterol [mg/100 g]	pH	Cholesterol assimilation [%]
Control	470.363 ± 0.2832	6.40	–
L. casei subsp. *casei* BK10–48	373.375 ± 0.010	4.36	20.62
L. intestinalis AC18–85	275.662 ± 0.2109	4.00	41.40
L. agilis BK10–47	272.525 ± 0.0571	4.03	42.06
L. agilis AB7–35	317.388 ± 0.1394	3.81	32.52
E. faecalis BC21–104	264.788 ± 0.2449	4.38	43.71
L. casei subsp. *casei* AB16–65	270.838 ± 0.0414	3.84	42.42
L. maltaromicus A21–101	189.800 ± 0.0467	4.36	59.65
L. maltaromicus AC3–16	189.186 ± 0.3221	3.76	59.78
L. agilis AC3–10	265.825 ± 0.1058	3.99	43.49
E. malodaratus BK9–43	368.975 ± 0.2968	4.18	21.56

Tab. 3. Quantity of cholesterol in butter, pH and ratio of cholesterol assimilation.

	Lactic acid bacteria	pH	Quantity of cholesterol [mg/100 g]	Cholesterol assimilation [%]
	Control	6.11	205.4	–
Run 1	*L. maltaromicus* AC3–16	4.46	205.12	0.1339
	L. casei subsp. *casei* AB16–65	4.60	197.07	4.0531
	Control	5.26	250.33	–
Run 2	*L. maltaromicus* AC3–16	3.87	201.8	19.38
	L. casei subsp. *casei* AB16–65	3.96	186.88	25.35

It was observed that the strains AC 3–16 and AB16–65 in broth and cream exhibited different abilities to remove cholesterol as the pH values were altered. As can be seen from Tab. 3, *L. maltaromicus* AC 3–16 reduces cholesterol by 0.1339% when the pH value is 4.46, whereas this is increased to 19.38% when the pH is reduced to a level of 3.87. In a similar way, cholesterol absorption by *L. casei* subsp. *casei* AB16–65 increases from 4.0531 to 25.35% when the pH value is decreased from 4.60 to 3.96. Pereira and Gibson [23] suggested that the cholesterol assimilation ability of the bacteria is highly dependent on their growth, in each run perhaps reflecting the growth stage of the inoculum used.

In the present study, the assimilation ratios of bacteria are found to be similar in broth and cream. Statistical analysis indicated no significant difference ($p > 0.05$) in the assimilation ratios in cream and MRS-THIO broth. But in butter, when the data was investigated, the cholesterol assimilation ratio was lesser than in both broth and cream.

Lactic acid bacteria and probiotic bacteria are frequently associated with health-promoting effects in human and animal intestines. Several studies show that there is a relation between consumption of cultured dairy products and a reduction of serum cholesterol levels in humans and animals, and some lactic bacteria have hypocholesterolemic effects [24, 25]. The hypocholesterolemic effects of some bacteria were attributed to their ability to bind cholesterol in the small intestine [26].

Cholesterol assimilation of some lactic acid bacteria has been reported in several studies [25, 27, 28]. According to Gilliland *et al*. [25], cholesterol was assimilated by *L. acidophilus via* the cells. Klaver and van der Meer [28] reported that assimilation of cholesterol by *L. acidophilus* was due to the coprecipitation of cholesterol along with free bile acids by the lactobacilli during growth. Hosono and Ton-oka [29] reported that lactic acid bacteria bind cholesterol to the cells and they suggested that this binding of cholesterol to the cells may be a physical phenomenon related to the cell wall. It was reported that some strains of *L. acidophilus* incorporated some of the cholesterol into their cellular membrane [21–30].

We do not believe that cholesterol merely coprecipitates with free bile acids, as has been indicated by Klaver and van der Meer [28]. This is because cream and butter have no bile acids, yet cholesterol assimilation was observed in both of them. The greatest role seems to be played by the bacteria strains. The difference between the assimilation ratios in cream and butter is thought to arise from the difference of their fat content. Juskiewicz and Panfil-Kuncewicz [4] reported that a higher degree of cholesterol content reduction was observed in products of lower fat content. Most of these studies were done *in vitro*; however, in the present study, cholesterol assimilation was applied to a fermented dairy product. In this field there is very little published work. It is clear that further work is required for a deeper understanding of this phenomenon in this field.

4 Conclusions

The results from the present study clearly indicate that probiotic bacteria applied to cream and butter, as well as to broth, caused a reduction of the cholesterol content in the product in relation to its fat content. The degree of cholesterol reduction depended greatly on the bacteria strain. A higher degree of cholesterol reduction was observed in broth and cream (15% fat) than in butter (which made 35% fat cream). When butter is produced by using a culture with a high assimilation ratio, not only a reduction of cholesterol in butter is observed but also the assimilation of cholesterol in the intestine is realized. That is why the probiotic properties of the bacteria are important. Thus, a combination of probiotic cultures such as *L. maltaramicus* AC 3–16 and *L. casei* subsp. *casei* AB16–65 could be used to manufacture soured butter that would have enhanced hypocholesterolemic activity; however, it requires further research in this field.

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