The Properties of *Enterococcus faecium* and the Fermented Milk Product—Gaio®

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3.1 INTRODUCTION

Many fermented foods are produced throughout the world. Fermentation is a process that transforms the starting material into a product that may have enhanced nutritional and/or organoleptic characteristics. With the advent of probiotics, many researchers have analyzed the microflora in traditional fermented foods in attempts to find foods that contain bacteria that may be beneficial to health, metabolism, and disease resistance. In a few cases, an opposite approach has been taken. Based on studies testing individual bacteria in animals and humans, new products have been developed that include these bacteria, thereby creating new probiotic foods. (See Chapter 6 on LcS for such an example.)

Early studies on *Enterococcus faecium* and its effects against diarrhea and, more importantly, on cholesterol metabolism showed that *E. faecium* might be an ideal candidate to include in a fermented milk probiotic product. Gaio (which contains both *E. faecium* and *Streptococcus thermophilus*) was developed and is now distributed in at least two European countries. This chapter reviews studies where *E. faecium* and Gaio were tested for their effects on serum cholesterol, diarrhea, and mutagens.

3.2 SERUM CHOLESTEROL AND CARDIOVASCULAR DISEASE

3.2.1 BACKGROUND

Coronary artery disease is one of the most frequent causes of morbidity and mortality all over the world.\(^1\) In the United States, it accounts for fully one-half of the nearly one million deaths each year from cardiovascular disease and is the leading cause of death for both genders.\(^2\) Each year, about 1.5 million Americans suffer acute myocardial infarction, and almost all myocardial infarctions are due to atherosclerosis of the coronary arteries.\(^3\) It is known that individuals with some conditions, designated as risk factors, have a higher chance in prematurely developing this disease. Among the risk factors, hypercholesterolemia is one of the most important. A continuous and graded positive relation was demonstrated between serum total cholesterol level and coronary artery disease mortality in the more than 350,000 men screened for the Multiple Risk Factor Intervention Trial (MRFIT).\(^4\) However, the numbers of people with very high serum cholesterol levels are not expressive, so they do not account for a large number of cases of symptomatic coronary disease. The vast majority of these cases are individuals presenting cholesterol considered to be in the normal (average) range or with a slight increase.\(^5,6\) According to the National Cholesterol Education Program—Adult Treatment Panel III,\(^7\) the optimal levels in a human blood lipid profile are total cholesterol below 200 mg/dL, low-density lipoprotein (LDL) cholesterol below 100 mg/dL, high-density lipoprotein (HDL) cholesterol above 40 mg/dL and triglycerides below 150 mg/dL. Such patients, in general, also have other risk factors, including smoking, hypertension, diabetes mellitus, sedentary life, weight excess, and psychosocial stress, because of the modern lifestyle typical in western industrialized countries.

It was demonstrated that healthy people presenting serum cholesterol levels within the normal range have a reduction in the risk of future cardiovascular events when their cholesterol levels are decreased.\(^8\) One metaanalysis performed showed
that for a 10% cholesterol reduction, the mortality risk due to cardiovascular disease is decreased by 15%, and total mortality risk is decreased by 11%. Consequently, any dietary intervention that could help to decrease serum cholesterol levels, particularly in people who do not have highly elevated levels, probably will be helpful in the prevention of coronary heart disease.

### 3.2.2 Cardiovascular Disease and Probiotics

Modulation of the microbial community (approximately $10^{14}$ bacterial cells/g) in the gut by probiotics and prebiotic foodstuffs has been considered as an important opportunity to positively influence human health. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon and thus improve health. Probiotics are defined as live microbial food ingredients that are beneficial to health.

The question of whether lactic acid bacteria (LAB) can be beneficial to health was raised a long time ago. Metchnikoff was the first to show an interest in LAB and their possible beneficial effects on health. He claimed that lactobacilli from fermented milk were able to prolong life through a reduced formation of toxins in the gut. Mann and Spoerry described a hypocholesterolemic effect of fermented milk in the natives of the African Masai tribe. These authors proposed that dietary habits were the explanation for the low incidence of ischemic heart disease among these people, because they traditionally had a high intake of meat, milk, and fermented milk. This meant that they also were consuming large quantities of saturated fat and cholesterol. After these seminal observations, many studies addressed this topic, as has been reviewed. Until now, no clear answer has been available, and even more difficult is the identification of a possible biochemical mechanism that can explain how this improvement of health is really achieved. One possibility is that milk and fermented milk products have an effect on the metabolism of serum lipids and lipoproteins, resulting in a better serum lipid profile. Not all studies have come to the same conclusion, as discussed by Eichholzer and Stähelin in an extensive review of this subject, and more recently by the Mission Scientifique de Syndifrais and by St-Onge et al. A controversy exists because milk and milk products have been shown to be atherogenic due to their concentration of saturated fat and cholesterol, but they also possibly play a role in the prevention of coronary heart disease. There appear to be scientific reports to support both effects. Other benefits of milk products such as yogurt have been reported, including beneficial intestinal flora changes, better immunological response, production of antibiotic substances, improved calcium absorption, prevention of osteoporosis, cataract prevention, and prolongation of life, that would justify increased consumption of milk products.

A group at Kiev University (unpublished results) demonstrated a significant hypocholesterolemic effect utilizing a milk product fermented with bacteria isolated from Abkhasia in the Caucasus. This region has a reputation for the longevity of its people, and it is known that fermented milk is a major part of the traditional diet of this population, suggesting a link between these two observations.
The studies analyzed in this chapter utilize a yogurt-like product, similar to the one used in the study of Kiev University, but produced in Denmark. The product Causido® (Gaio) was constituted as a fermented (probiotic) dairy product, containing a bacterial culture (*E. faecium, S. thermophilus*) consumed for different periods of time.

### 3.3 ENTEROCOCCUS FAECIUM

#### 3.3.1 CHARACTERISTICS AND OCCURRENCE

Enterococci are included in the broad category of LAB. Enterococci are Gram-positive, nonsporeforming, catalase-negative, oxidase-negative, coccus-shaped bacteria, and occur singly, in pairs, or in chains. The identification of the members of the genus *Enterococcus* using traditional classification tests is difficult, because there are no phenotypic characteristics that can be used to distinguish them from other closely related bacteria. Enterococci are generally considered as being hardy because they survive in a wide range of temperatures, pH levels, saline solutions, and environments, such as are found in the human gastrointestinal tract. An important distinguishing characteristic (from a human health perspective) of various enterococci is their ability to resist many antibiotics.

Enterococci occur on plants and in the feces of animals and human. *Enterococcus faecalis* and *E. faecium* are the two most common species found in human gastrointestinal tracts. *E. faecalis* counts can reach $10^5$ to $10^7$ colony-forming units (CFU)/g, while *E. faecium* levels of $10^4$ to $10^5$ CFU/g are found. Levels of these two species can vary among individuals; diet and other factors are believed to alter the proportions of *Enterococcus* species. *E. faecalis* has been isolated in feces of neonates, but *E. faecium* has not.

#### 3.3.2 ANTIBIOTIC RESISTANCE

Many authors have presented criteria that should be used when choosing potential probiotic bacteria. Above all, it is agreed that any microorganism that is intentionally added to a food should be generally recognized as safe (GRAS). For example, the microorganism should not be pathogenic, should not produce toxins or metabolites that could adversely affect the health and metabolism of the host, and should not negatively impact on bacterial populations already resident in the host. A large number of fermented food products that contain LAB are now sold because they have a long history of being safe. Within the LAB, enterococci and streptococci have been identified as causes for concern. With the introduction of a fermented milk product on the market that contains *E. faecium*, the question of antibiotic resistance has been raised.

Plasmids are genetic elements independent of the cell chromosome. Over time, some plasmids have developed genes that make them resistant to selected antibiotics. Plasmids are easily transferred between cells, and this allows the efficient spread between bacteria of resistance to an antibiotic. Antibiotics originally were substances produced by fungal microorganisms to eliminate competition from bacteria for survival reasons. However, the number of antibiotics now available and...
their widespread use in prescription drugs and for general disinfection purposes has raised concerns about the potential for the development of antibiotic resistance. The introduction of probiotic foods into the diet raises the possibility of the ingested probiotic becoming antibiotic-resistant from related intestinal bacteria that already have acquired resistance, or more seriously, the reverse—a probiotic product containing antibiotic-resistant bacteria that pass antimicrobial resistance genes or genes that encode for virulence factors onto resident bacteria.

Most strains of enterococci are resistant to tetracycline, erythromycin, clindamycin, chloramphenicol, and sulfonamides.27 This, together with the fact that the incidence of infections attributed to enterococci appears to be increasing and along with the difficulty in treating such infections, places these organisms as important human pathogens of concern. The hospital environment is one where enterococcal contamination has received much attention. However, because vancomycin-resistant enterococci (VRE), in particular, have been identified in a wide variety of farm animals and birds, it is not clear whether the food is a major vector in the transfer of VRE.34

With respect to Gaio, Lund et al.35 were able to show that when Gaio was taken together with vancomycin, the total number of enterococci decreased at day 10 of the feeding trial, but by 3 weeks after the cessation of the experiment, enterococci numbers were 100 times those counted at the beginning of the experiment. Subjects having received only the Gaio had no such increase in enterococci numbers, indicating that the antibiotic treatment may have given the ingested probiotic bacteria in the Gaio an advantage due to reduced colonization resistance. No major overgrowth of VRE occurred in any subjects, including those receiving the vancomycin treatment. However, some subjects (20%) were transient carriers of VRE. Resistant strains isolated in subjects were not associated with the consumption of Gaio, whereas pulse-field gel electrophoresis analysis showed that the strains of *E. faecium* found in Gaio, and VRE fecal samples were different. Lund et al. were able to state that “no resistance against vancomycin emerged in intestinal enterococci, and *E. faecium* from the Gaio product was not found to acquire vancomycin resistance during the study period.”

In a companion paper that used some of the samples from their 2000 study,35 Lund et al.36 used genotypic and phenotypic analyses to confirm that *E. faecium* found in Gaio can survive GI tract transit, and that during the consumption period of the experiment, Gaio-derived *E. faecium* became the dominant part of the total *E. faecium* in the gut. However, the Gaio-derived *E. faecium* was not found in fecal samples of subjects receiving both the Gaio and vancomycin, and it did not persist 3 weeks after consumption was stopped.

Adams37 pointed out that there have been no reports of infection contracted as a result of consumption of foods containing enterococci. However, the Lactic Acid Bacteria Industrial Platform recommended that enterococci should not be used in foods unless there was a demonstrable (possibly health) benefit.38

### 3.4 Production of Gaio

Gaio was first produced by the Danish dairy corporation MD Foods A/S established in Aarhus in Denmark. Recently, MD Foods merged with Arla Foods, and the product is only produced by Arla Foods and consumed in Denmark and Sweden.
The production of Gaio uses a fermentation of milk at a temperature of 37°C. The level of starter inoculated is approximately $5 \times 10^{12}$ CFU/1000 L of milk. The fermentation time is approximately 9 h, to a final pH of 4.5. The final product is very viscous and has a mild, slightly acid taste. The product is sold in plastic containers of 500 g as “natural” and with different fruit flavors. The product is distributed and sold refrigerated.

The original Ukrainian bacterial culture (Causido) is used to produce Gaio. This culture contains one human species of *E. faecium* and two strains of *S. thermophilus*. The CFUs of the fresh product are $10^5$ to $10^9$/mL for *E. faecium* and 5 to $20 \times 10^8$/mL for *S. thermophilus*. One hundred grams of the product has an energy content of 240 kJ and contains 4.9 g of protein, 5.4 g of carbohydrate, and 1.5 g of fat (66% as milk fat and 33% as soybean fat). The cholesterol content is about 5 mg for every 100 g of the product. Vitamins E and C are added in accordance with the original Ukrainian recipe, giving final concentrations of 0.5 mg and 10 mg, respectively, per 100 g.

### 3.5 CHOLESTEROL EXPERIMENTS

#### 3.5.1 Early Work

The observation that the Masai people had low serum cholesterol levels in spite of their high dietary cholesterol intakes\(^{15}\) initiated interest in the beneficial effects of fermented milk on cholesterol metabolism, because the Masai consumed large quantities of fermented cows’ milk. Several studies were then carried out, with mixed results, in animal models (rat, rabbit) and humans to investigate the properties of milk fermented with a variety of bacteria on cholesterol metabolism.\(^{39-41}\)

Zacconi et al.\(^{42}\) used axenic mice to show that when mice were fed with a hypercholesterolemic diet for 60 d, animals reared in sterile boxes had higher serum cholesterol levels than those dosed with various bacteria. Although the quantity of bacteria given to each animal was not indicated, it was evident that the largest reductions in serum cholesterol levels occurred in mice given *E. faecium* (females: –16.9%; males: –7.8%) and *Lb. acidophilus* (females: –11.4%; males: –5.3%). Zacconi et al. also found that the reductions were greatest in the female mice, and that the animals receiving the *E. faecium* were healthier than the axenic mice.

#### 3.5.2 Studies with *Enterococcus faecium*

Mikeš et al.\(^{43}\) carried out a human-feeding trial in which subjects were given lyophilized *E. faecium* M-74 ($5 \times 10^9$/d) in capsules for 6 weeks. The mean number of *E. faecium* in fecal samples generally rose and plateaued during the period of dosing, and then fell slowly during the following 5 weeks. However, large individual differences in the numbers of *E. faecium* recovered in fecal samples were noted. Fecal ß-D-glucuronidase activity was measured during the experiment, and it was found that activity decreased during the dosing period and remained low 5 weeks after the dosing with *E. faecium* was stopped. The serum LDL and total serum cholesterol rose during the initial weeks of the study; both parameters dropped significantly (compared to values obtained before the study) 14 d after the consumption of the
bacteria was discontinued. Conversely, serum HDL values rose after the bacteria treatment stopped. There were no changes in other blood parameters.

To better understand how the bacteria might be altering cholesterol metabolism, the metabolic activation of neutrophils was measured, because it is known that neutrophils are capable of producing reactive oxygen intermediates that can oxidize lipoproteins and thereby contribute to atherosclerosis. Mikeš et al. found that during the time when LDL and total cholesterol were lowered, stimulated neutrophils from subjects had increased ability to reduce 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and to produce superoxide. This finding was consistent with an earlier observation that *E. faecium* was able to stimulate human peripheral neutrophils resulting in the production of reactive oxygen intermediates in vitro. The observed reciprocal correlation between cholesterol levels and neutrophil INT reductase activity and superoxide production raised the possibility that the *E. faecium* might be affecting some oxidative process, which in turn reduces cholesterol levels.

In a recent human trial (double blind, placebo controlled) that lasted over 1 yr, Hlivak et al. reported that daily consumption of capsules containing *E. faecium* M-74 (2 × 10⁹ CFU/capsule) and selenium (30 μg/capsule, organically bound) produced a gradual decline in serum total cholesterol that was significantly different from the day 0 value after 3 weeks, and that persisted 4 weeks after the cessation of treatment. The total serum cholesterol for the placebo group also had a gradual, but not statistically significant decrease over the course of the experiment. LDL values declined in a pattern similar to total cholesterol; the *E. faecium* group had significantly lower LDL values one month after the experiment compared to day 0 values. HDL and triglyceride values did not change in the placebo group, but the *E. faecium* group had significantly reduced HDL after 12 weeks. This latter effect was attributed to activation of inflammation processes that can reduce the size of HDL particles. In a companion paper, Hlivak et al. presented data from this same clinical trial to explain how the consumption of *E. faecium* M-74 may help prevent and treat cardiovascular disease. Compared to day 0, subjects receiving daily capsules of *E. faecium* M-74 had significantly reduced serum levels of the cell adhesion molecule sICAM-1, and significantly decreased expression levels of the surface adhesion molecules CD11b on lymphocytes, and CD31 and CD54 on granulocytes. Paradoxically, significant increases were also measured for the expression of some other surface adhesion molecules. Hlivak et al. stated that studies at the molecular and genetic level could be used to explain and predict atherosclerosis.

### 3.5.3 Studies with *Streptococcus thermophillus*

*S. thermophilus* and *Lactobacillus bulgaricus* are the two bacteria added to milk to produce yogurt. The effect, if any, of *S. thermophilus* on serum cholesterol is not clear because most feeding trials with animals and humans have tested *S. thermophilus* in combination with other LAB. For example, Akalin et al. reported a study where they fed mice a chow diet and yogurt containing either *S. thermophilus* and *Lb. delbrueckii* ssp. Bulgaricus or *S. thermophilus* and *Lb. acidophilus*. The fresh yogurt had 10⁷ lactobacilli/mL. The number of *S. thermophilus* in the yogurt was not measured. The animals eating the yogurts had lower serum cholesterol levels and LDL cholesterol...
(significantly lower in the case of *S. thermophilus* and *Lb. acidophilus*) than control mice that ate chow. Serum triglycerides did not appear to be affected. These authors concluded that the effects on cholesterol metabolism were attributable to the *Lb. acidophilus* in the yogurt and that the *S. thermophilus* had no effect.

Kawase et al.\(^4^8\) showed that *Lactobacillus casei* TM0409, *S. thermophilus* TMC1543, and whey protein concentrate had a synergistic effect on lowering serum cholesterol in rats. They then used the two bacteria to produce a fermented milk that contained \(6.1 \times 10^8\) *Lb. casei*/mL and \(2.6 \times 10^7\) *S. thermophilus*/mL and fed it to male human volunteers for 8 weeks (200 mL, twice a day). HDL cholesterol was significantly increased after 4 weeks, and the increase continued until week 8. Total serum cholesterol was lower, but not statistically lower, in the group receiving the fermented milk compared to the group receiving a placebo. No mechanism was proposed to explain the hypocholesterolemic effect of the fermented milk.

### 3.5.4 Human Trials with Gaio

The first study to utilize a milk product fermented with bacteria isolated from Abkhasia was carried out by Sarkisov at Kiev University in Ukraine. However, the results were not published. These authors showed a cholesterol-lowering effect of approximately 39 mg/dL, an increment in HDL cholesterol, and a reduction in triglycerides after 4 weeks of dietary supplementation with the new product in a very heterogeneous group of males and females.\(^4^9\)

Four studies in which the effect of Gaio consumption on blood lipid profiles was evaluated can be found in the literature. The first published trial to test the effects of Gaio (a product practically identical with the Ukrainian one, though produced in Denmark), on LDL cholesterol level was performed by Agerbaek et al.\(^5^0\) They studied 58 male volunteers of Danish descent, all 44 yr old, with normal cholesterol fasting values. All were selected from a cohort examined in 1989 and again in 1990 in a study of the prevalence of risk factors for coronary heart disease at the University Hospital of Aarhus. They were selected on the basis of having had normal fasting values of serum cholesterol (5.0 – 6.5 mmol/L) and triglycerides (< 5.0 mmol/L) at both examinations, no history of cardiovascular, cerebrovascular, or metabolic disease; normal weight (body mass index [BMI] < 27.5) with a stable weight; alcohol consumption < 315 g/week; and normal blood pressure (< 150/95 mmHg). During the intervention period, the subjects maintained their habitual diets supplemented with 200 mL/d of either Gaio or a similar placebo product (chemically fermented). Fasting blood samples were drawn initially and after 3 and 6 weeks and analyzed for plasma values of total cholesterol, HDL cholesterol, and triglycerides. LDL cholesterol was estimated by the Friedewald formula \([\text{LDL cholesterol} = \text{total cholesterol} – \text{HDL cholesterol} – \text{VLDL cholesterol (triglycerides/5)}]\).\(^5^1\) After 6 weeks, the consumption of Gaio produced an average reduction of 6% in total serum cholesterol levels, completely ascribed to a decrease in LDL cholesterol level of 10%, whereas HDL cholesterol and triglycerides were unchanged. The authors stated that although the findings were promising, the results could not be extrapolated to other human subjects who were not included in their study. They suggested further studies to determine the potential effects of the new fermented milk product on lipoproteins.
among women, the elderly, and in subjects with manifest hypercholesterolemia. They also stated that the potential beneficial effect for middle-aged men would be true only if the cholesterol-lowering effect persisted for longer periods than the 6 weeks investigated in their study. This is based in the findings of prevention studies using drugs. Those studies showed that improvement in vascular risk only begins to appear after 1 to 2 yr of drug use and cholesterol reduction.9

Following this line of reasoning, Richelsen et al.52 studied the consumption of Gaio for a longer period of time in a randomized, double-blind, and placebo-controlled trial that included 87 nonobese and normocholesterolemic females and males, aged 50 to 70 yr. The volunteers were recruited through announcement in the local newspaper. Before inclusion in the experiment, blood samples were drawn to eliminate subjects with liver disease, diabetes, kidney disease, anaemia, and hypercholesterolemia (total cholesterol > 8.0 mmol/L). Inclusion criteria were healthy men and women aged 50 to 70 yr (the women were all postmenopausal), body mass index < 32 kg/m², and no medication influencing plasma lipids. Participants were instructed not to change their ordinary diet, alcohol intake, level of physical exercise, and tobacco consumption during the study period. They consumed 200 mL of either the fermented milk product (Gaio) or a placebo (chemically fermented). The study showed a rapid reduction in LDL cholesterol level by about 8% after 1 month, but after 6 months, although the effect remained, the reduction in serum LDL cholesterol was similar to the reduction observed in the placebo group. The authors reasoned that after 1 month of the placebo use there was a gradual fall in total and LDL cholesterol in both genders, making the interpretation of the results less clear-cut. Thus, after 6 months the levels of total cholesterol and LDL cholesterol were significantly lower than initial values in both groups, but the reduction and the absolute values were not different in the two groups (treatment versus placebo). They speculated that the placebo product itself, without the bacterial culture but chemically fermented with an organic acid (gluconic acid-delta-lactone) containing 1.5% fat, could have cholesterol-lowering effects or, alternatively, that the seasonal variation in subjects’ lipid levels53 could explain the results. The authors also suggested that some people participating in the trial could be “responders,” whereas others could be “nonresponders,” but the basis for this biological (genetic) phenomenon is still unknown. Another possibility suggested by the authors was that the absence of effect could be due to the lack of statistical power to detect a 4 to 5% reduction in blood cholesterol due to the small number of subjects included in the study.

Bertolami et al.54 tested the effect of Gaio in a group of patients with primary hypercholesterolemia (11 men and 21 women, 36 to 65 yr old) who had not shown a significant improvement in LDL cholesterol level after dietetic modifications alone (Phase I of the American Heart Association–National Cholesterol Education Program [NCEP] Adult Treatment Panel II). A prospective, randomized, double-blind, 8-week crossover design, controlled by placebo (a chemically fermented milk) was used. After initial clinical and laboratory analysis, the patients began to consume 200 g daily of Gaio or the placebo in a randomized and double-blind manner. Seventeen patients started the trial using the active product and fifteen began with placebo. After 8 weeks, blood was collected again for lipid profile evaluation and the crossover was made (those consuming Gaio changed to placebo and vice versa).
After an additional 8-week period, blood was collected for the last lipid profile determination. The results showed that Gaio was able to significantly reduce total cholesterol by 5.3% and LDL cholesterol by 6.15% in these hypercholesterolemic subjects, compared with the placebo product. Like Richelsen et al.,52 Bertolami et al. also suggested the possibility of different patient responses (“responders” and “non-responders”) to the use of Gaio and the placebo product (see Tables 3.1 and 3.2).54

### TABLE 3.1

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Phase 1 (Diet only)</th>
<th>Phase 2 (Diet + placebo)</th>
<th>Phase 3 (Diet + Gaio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>248.47 (± 26.75)</td>
<td>249.09 (± 28.45)</td>
<td>235.75 (± 35.03)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>119.16 (± 49.28)</td>
<td>118.38 (± 39.47)</td>
<td>116.66 (± 38.79)</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>52.38 (± 14.00)</td>
<td>56.91 (± 15.96)</td>
<td>54.41 (± 14.97)</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>172.22 (± 21.17)</td>
<td>168.59 (± 24.18)</td>
<td>158.00 (± 31.04)</td>
</tr>
<tr>
<td>Weight</td>
<td>66.55 (± 10.57)</td>
<td>66.05 (± 10.37)</td>
<td>65.97 (± 10.60)</td>
</tr>
</tbody>
</table>

Note: Significant difference between phases: a Comparison between averages observed after phases 1 (diet only) and 3 (active product) – \( P = 0.012 \); b Comparison between averages observed after phases 2 (placebo) and 3 (active product) – \( P = 0.004 \); c Comparison between averages observed after phases 1 (diet only) and 2 (placebo) – \( P = 0.001 \); d Comparison between averages observed after phases 1 (diet only) and 3 (active product) – \( P = 0.002 \); e Comparison between averages observed after phases 2 (placebo) and 3 (active product) – \( P = 0.012 \); f Comparison between averages observed after phases 1 (diet only) and 2 (placebo) – \( P = 0.026 \); g Comparison between averages observed after phases 1 (diet only) and 3 (active product) – \( P = 0.014 \).


### TABLE 3.2

<table>
<thead>
<tr>
<th>Cholesterol</th>
<th>Phase comparison</th>
<th>Mean (SD)</th>
<th>Maximum decrease</th>
<th>Maximum increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>D × P</td>
<td>+ 0.39 (6.82)</td>
<td>− 11.38</td>
<td>+ 13.45</td>
</tr>
<tr>
<td></td>
<td>D × Y</td>
<td>− 5.04 (10.44)</td>
<td>− 24.26</td>
<td>+ 23.10</td>
</tr>
<tr>
<td></td>
<td>P × Y</td>
<td>− 5.30 (9.38)</td>
<td>− 23.40</td>
<td>+ 18.77</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>D × P</td>
<td>− 1.90 (9.39)</td>
<td>− 19.87</td>
<td>+ 16.02</td>
</tr>
<tr>
<td></td>
<td>D × Y</td>
<td>− 8.29 (13.29)</td>
<td>− 37.69</td>
<td>+ 23.78</td>
</tr>
<tr>
<td></td>
<td>P × Y</td>
<td>− 6.15 (12.73)</td>
<td>− 30.00</td>
<td>+ 18.65</td>
</tr>
</tbody>
</table>

Note: D = diet only; P = diet plus placebo; Y = diet plus yogurt; SD = standard deviation.

Enterococcus faecium and the Fermented Milk Product—Gaio

Agerholm-Larsen\textsuperscript{55} studied the effects of Gaio in 70 overweight and obese subjects in a randomized, double-blind, placebo- and compliance-controlled, parallel protocol. The study was designed as a five-armed parallel study in which Gaio was compared with two other fermented milk products, a chemically acidified milk product with an organic acid (delta-acid-lactone) instead of a living bacterial culture, and an inert placebo pill. One comparison milk product was fermented with two strains of \textit{S. thermophilus} (~ 1 × 10\textsuperscript{7} CFU/mL) and two strains of \textit{Lb. acidophilus} (~ 2 × 10\textsuperscript{7} CFU/mL), and the other was fermented with two strains of \textit{S. thermophilus} (~ 8 × 10\textsuperscript{8} CFU/mL) and one strain of \textit{Lb. rhamnosus} (~ 2 × 10\textsuperscript{8} CFU/mL).

The protocol involved, besides lipid level evaluation, the determination of fibrinogen and C-reactive protein because these two acute-phase proteins are also implicated as risk factors for coronary artery disease in healthy men.\textsuperscript{56,57} The authors planned to offer an increased amount of fermented milk product (450 mL daily) to obtain a greater effect on LDL cholesterol. The 20 men and 50 women participating were healthy, weight-stable, overweight and obese (25.0 < BMI < 37.5 kg/m\textsuperscript{2}), 18 to 55 yr old. Participants were instructed not to change their habitual diet, level of physical exercise, tobacco and alcohol habits, or their body weight during the study period. Compliance was tested at home every second week (weeks 2, 4, 6, and 8) by analyzing sample yogurt bags labelled with \textsuperscript{13}C-acetate by a nondispersive infrared spectroscopy method. After 4 weeks of consuming 450 mL of Gaio a day, there was no decrease in LDL cholesterol levels. After 8 weeks these levels decreased significantly by 8.4%, and fibrinogen increased also significantly compared to the placebo group, whereas C-reactive protein did not change. The authors expected to see differences in lipids after 4 weeks as demonstrated in previous comparable studies but found none. Although they had no obvious explanation for the lack of reduction in LDL cholesterol levels after 4 weeks, they speculated that a possible mechanism behind this finding could perhaps be the small number of subjects in each group.

According to the authors, it is likely that there is some between-subject variability in intestinal colonization of the active bacteria that could influence these results. As fibrinogen is an acute-phase protein, the authors speculated that the increased fibrinogen concentration found in the Gaio group could be attributed to immunostimulation. They also suggested that it was not possible to exclude the idea that a transient colonic inflammation caused by the bacterial strains in Gaio was the reason for the increase in fibrinogen in subjects consuming this fermented milk product. However, the lack of any increase in C-reactive protein does not support this possibility.

### 3.6 METAANALYSIS OF CHOLESTEROL EXPERIMENTS

In a published metaanalysis, Agerholm-Larsen et al.\textsuperscript{58} questioned the conflicting results pertaining to this product’s efficacy in reducing plasma cholesterol. They analyzed six studies conducted with Gaio involving 425 subjects of both genders and different initial cholesterol levels, concluding that five studies showed a small beneficial short-term effect of 6 to 10% on serum LDL cholesterol (Sarkisov et al.,\textsuperscript{49} Agerbaek et al.,\textsuperscript{50} Richelsen et al.,\textsuperscript{52} Bertolami et al.,\textsuperscript{54} Agerholm-Larsen et al.\textsuperscript{55}). However, the long-term effect was inconclusive (Richelsen et al.\textsuperscript{52}), and one study failed to demonstrate any effect at all (Sessions et al.\textsuperscript{59}). The authors pointed out
that a metaanalysis suffers from unpublished data material because negative studies often remain unpublished, leading to publication bias. However, they confirmed that they had no knowledge of other unpublished material on the Causido culture. As a conclusion, they suggested that the metaanalysis based on the five controlled study interventions showed that the intake of the fermented milk product (Gaio) produced a statistically significant and clinically important reduction in plasma cholesterol. They found a reduction of 5% in LDL cholesterol, which is considered large enough to have a beneficial effect on risk factors for coronary heart disease.\(^9\) However, they emphasized that long-term studies on Causido are required to document whether a sustained effect on the blood lipids occurs.

A summary of the results of the studies evaluating the effects of consumption of Gaio on plasma lipid profiles is shown in Table 3.3. Studies are listed by author, type of population enrolled (normo- or hypercholesterolemic), and the percent changes of total cholesterol and LDL cholesterol comparing active treatment versus placebo (only the study from Sarkisov was open and did not involve a placebo control).

### 3.7 MECHANISM OF ACTION

The reason for the observed hypocholesterolemic effect in subjects consuming Gaio is not fully understood at this time. It has been proposed that the cholesterol-lowering effect is related to the bacterial culture in the product.\(^{50}\) A potential explanation given by Agerholm-Larsen et al.\(^{55}\) is that there is an association between the gut microflora and cholesterol absorption in the small intestine. The intestinal bacteria can bind bile acids to cholesterol, resulting in the excretion of bile acid-cholesterol complexes in the feces. Decreased bile acid recycling through the enterohepatic circulation would result in cholesterol uptake from the circulation into the liver for de novo synthesis of bile acids. Another possible explanation was provided by St-Onge et al.\(^{22}\) High numbers of bacteria in products such as yogurt when consumed will ensure passage of sufficient numbers of bacteria into the intestine to exert effects on metabolism. Because

<table>
<thead>
<tr>
<th>Study</th>
<th>Study group</th>
<th>Percentage decrease in total cholesterol</th>
<th>Percentage decrease in LDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarkisov</td>
<td>Hypercholesterolaemic</td>
<td>15.87</td>
<td>16.85</td>
</tr>
<tr>
<td>(unpublished results, 1991)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agerbaek et al. (1995)</td>
<td>Normocholesterolaemic</td>
<td>6.08</td>
<td>9.77</td>
</tr>
<tr>
<td>Richelsen et al. (1996)</td>
<td>Normocholesterolaemic</td>
<td>3.51</td>
<td>5.96</td>
</tr>
<tr>
<td></td>
<td>(not significant)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sessions et al. (1998)</td>
<td>Hypercholesterolaemic</td>
<td>0.17</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>(not significant)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bertolami et al. (1999)</td>
<td>Hypercholesterolaemic</td>
<td>5.3</td>
<td>6.15</td>
</tr>
<tr>
<td>Agerholm-Larsen et al. (2000)</td>
<td>Normo-cholesterolaemic</td>
<td>5.14</td>
<td>6.64</td>
</tr>
</tbody>
</table>
the bacteria contained in fermented milk products are consumed with macronutrients that alter the stomach’s pH (buffering effect), bacterial survival is increased, and the bacteria pass into the small intestine and then into the large intestine. Once in the large intestine, the bacteria ferment indigestible carbohydrates and produce short-chain fatty acids. The relative proportions of this production are likely to alter cholesterol synthesis. Gaio contains *S. thermophilus* and *E. faecium*. In vitro studies have shown that *S. thermophilus* is acid sensitive and cannot survive the passage through to the small intestine. However, *E. faecium* is known to have good bile tolerance. Consequently, Agerholm-Larsen et al. suggest that *E. faecium* is the bacterial strain with the cholesterol-lowering effect.55

Rossi et al.60 showed that *E. faecium* has the ability to remove and assimilate cholesterol. Using a MRS medium supplemented with bile salts (Oxgall), 5 of 14 strains of *E. faecium* tested were able to reduce the concentration of suspended cholesterol (100 mg/L) by 30% or more, but only when bile salts were present in the MRS broth. There was an appearance of cholesterol in the bacterial cells that paralleled the decease of cholesterol in the MRS broth. Only 1 of 5 stains of *Lb. acidophilus* tested was able to reduce cholesterol concentrations. Using a similar protocol, the group then showed that *E. faecium* plus *Lb. jugurti* (1:1) not only reduced cholesterol by 43%, but could also be used to produce a fermented soymilk product with acceptable physiochemical and sensory attributes.61

### 3.8 OTHER PROPERTIES OF ENTEROCoccus FAECiUM

#### 3.8.1 Treatment of Diarrhea

One of the most obvious applications of probiotics is to restore the intestinal microflora population during diarrhea. A variety of LAB, including *Lb. rhamnosus* GG, *Lb. reuteri*, *Streptococcus boulardii*, and *E. faecium*, have been tested for their ability to reduce the severity or duration of various diarrheas.62 A group (78 people) of sufferers of acute diarrhea were found to have a lower frequency of diarrhea after 7 d of treatment with *E. faecium*, compared to those receiving a placebo.63 Results were not as positive in a 3 d study involving patients suffering from diarrhea due to infection with *Vibrio cholerae* (114 subjects) and enterotoxigenic *Escherichia coli* (41 subjects). In this study, there was no difference in the duration of the diarrhea between the placebo group and those receiving the *E. faecium*.64

#### 3.8.2 Antimutagenicity

Milk fermented with *E. faecium* has been investigated for its antimutagenic properties. Belicová et al.65 used an ether extract of milk that had been fermented with *E. faecium* to carry out a variety of tests. They reported that their milk extract showed a dose-dependent inhibition of mutagenesis induced by chemical and physical mutagens. Using both *Salmonella typhimurium* TA97 and TA100, they showed that the fermented milk extract (4 uL/plate) could reduce UV-irradiation damage by 72% and 55% (for TA97 and TA100, respectively). A 10 uL dose of extract produced about a 30% reduction in the mutagenicity of nitrovine; the same
dose reduced 5-nitro-2-furylacrylic acid mutagenicity by up to 25% and N-methyl-
N’-nitro-N-nitrosoguanidine (MNNG) mutagenicity by 28%.

Earlier, Ebringer et al. showed that viable Enterococcus faecium M-74 showed significant antimutagenic effects on nitrovin and 2-aminofluorene mutagenicity (Ames test) but heat-treated nonviable cells did not. They also reported that Enterococcus faecium cells themselves showed no immunostimulatory activity, but mixtures of Enterococcus faecium and phagocytes significantly stimulate mean INT-reductase activities. Ebringer et al. concluded that Enterococcus faecium contained factors that could reduce the effect of the mutagens they tested and that heat stable proteins might be responsible.

### 3.8.3 Cheese Making

Enterococci bacteria are commonly used in the production of raw ewes’ and goats’ milk cheeses. They contribute to proteolysis, lipolysis, and citrate breakdown, and thereby influence ripened cheese taste and flavor. Enterococcus faecium has been shown to be a good starter adjunct in the production of Cheddar cheese. Gardiner et al. showed that 9 months after adding $2 \times 10^7$ CFU/mL (0.1%) Enterococcus faecium PR88 to a commercial lactococcal starter, $3 \times 10^8$ CFU/g were viable. Cheese with the added bacteria was found to have increased proteolysis and higher levels of some odor-active compounds. The Cheddar cheese containing the Enterococcus faecium was judged (by a commercial grader) to be ripening faster and had a better flavor than the control cheese. This study confirmed previous reports about the positive effects of enterococci on ripening and flavor development in Cheddar cheese.

Enterococci bacteria have also been found on beef, poultry, and pig carcasses; cooked pork; and vegetables, especially olives. Their presence does not necessarily imply contamination by fecal material.

### 3.9 Conclusions

After analyzing the results of published studies results using Gaio as a possible option to obtain reduction in total and LDL cholesterol levels, it can be concluded that more studies are needed to ascertain:

- Whether the effects of prolonged consumption of Gaio on blood lipid profiles are the same as those observed during shorter periods of consumption, and
- Whether the final beneficial effect will lead to an improvement in coronary heart disease prevention with fewer events and fewer deaths due to this worldwide health problem of modern societies.

### References

Enterococcus faecium and the Fermented Milk Product—Gaio


