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Chapter

Cholesterol Uptake and Survival of *Lactococcus lactis* Strains in Fluids Simulating the Human Stomach and Duodenum

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Abstract

Scientific evidence exists showing that lactic acid bacteria, including the genus *Lactococcus*, have the capacity to bind and remove cholesterol. However, in many cases, in vivo and in vitro results are not unambiguous or reproducible; thus it appeared valid to conduct a study which would explain what factors determine adhesion and assimilation of cholesterol by *Lactococcus*. The study on *Lactococcus* bacteria under in vitro conditions in model digestive fluids may contribute to the explanation of the observed ambiguities. In vitro research has proven that *Lactococcus* is capable of removing free cholesterol under in vitro conditions in broths without bile salts, as well as in a simulated gastric fluid and in the conditions of simulated intestinal fluid. The amount of cholesterol removed by live cells of *Lactococcus* is directly proportionately dependent on the concentration of this substance, incubation temperature, count, and viability of cells. However, oftentimes these relationships are not linear. Under the conditions of model gastric fluid or intestinal fluid, the cultures of *Lactococcus* release portion of the previously bound cholesterol, independent of cell viability. The survival rate of *Lactococcus* cells in simulated gastric fluid or simulated intestinal fluid depends on the tested bacterial culture and does not depend on the presence of cholesterol.

Keywords: cholesterol, *Lactococcus*, survival, gastrointestinal tract, duodenum, gastric acid

1. Introduction

Coronary ischemia, known as the coronary disease, is one of the modern civilization diseases, whose cause is coronary atherosclerosis (so-called atherosclerotic coronary plaque) in over 90% of cases, leading to their stenosis. One of the risk factors for the formation of atherosclerotic coronary plaques is hypercholesterolemia, in particular elevated concentration of LDL cholesterol. Scientific data exist indicating that consumption of fermented milk products reduces the level of cholesterol in humans. Some of the studies (on animals and human volunteers) indicate that the reduction of the cholesterol level in blood serum is caused by lactic acid bacteria present in fermented milk drinks. Numerous in vitro studies demonstrate that the capacity to reduce the cholesterol level may be exhibited not only by the strains with

documented probiotic traits but also some "traditional" lactic acid bacteria used in the production of cheese, cream, or fermented milk products. The role of *Lactococcus* in dairy fermentation is mostly down to the production of lactic acid; however these bacteria utilize less than 0.5% of lactose from milk. Only *Lactococcus lactis* is applicable in the dairy industry, with its two subspecies: *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. These subspecies comprise the basic component of dairy mesophilic starter cultures, used for the production of cream, buttermilk, cottage cheeses, cheeses, and fermented milk [1–3]. In the process and functional terms, *Lactococcus* possess all the traits required for starter cultures: the capacity to ferment lactose, resistance to low pH, low temperature, and high concentrations of cooking salt. They are characterized by stability and suitable survival time during lyophilization and freezing and in the storage process of starter cultures [1, 2, 4].

Furthermore, lactic acid bacteria have the capacity to reduce the level of cholesterol in simulated conditions, i.e., culture media. It is known that lactic acid bacteria are not capable of metabolizing cholesterol, meaning its transformation into other compounds. It has been noted that bacterial cells are capable of binding cholesterol, consisting in adhesion of substances by the cell wall or its assimilation into cell wall. It has also been suspected that lactic acid bacteria are capable of deconjugating bile salts being the component of bile, followed by coprecipitation of cholesterol with deconjugated bile acids. Furthermore, tests on gnotobiotic animals demonstrated that hydrolysis of bile enhances its secretion and thus may contribute to reduction of the cholesterol level in blood serum. Moreover, the cholesterol level in the human organism may be also influenced by exopolysaccharides (EPS) produced by numerous lactic acid bacteria species. It is believed that these bacteria, similar to fiber, can bind cholesterol and bile acid molecules present in intestines and remove them from the human organism.

In many cases, results of in vitro studies are not unambiguous, or lack of their reproducibility has been determined. It turns out that also in vivo tests conducted on human volunteers or experimental animals do not produce unambiguous results or that their results are divergent. Considering that it is difficult to explain as to why this happens, such studies are frequently criticized for methodological and technical errors and lack of reproducibility.

2. Influence of lactic acid bacteria, including *Lactococcus*, on the cholesterol level in humans

The extensive collection of scientific publications devoted to health-promoting properties of lactic acid bacteria includes articles presenting studies on the possibility of reducing the cholesterol level in human and animal organisms through consumption of fermented milk products including traditional and probiotic strains of lactic acid bacteria.

As early as in 1974, Mann and Spoerry [5, 6] determined the reduced level of cholesterol in the blood serum of men from the African Maasai, which stemmed from the consumption of high amounts of fermented milk containing wild lactic acid bacteria strains. This research enabled researchers to look for the methods of reducing the cholesterol level in the human organism, although the first reports on the positive impact of fermented milk drinks on the reduction of the cholesterol level in live organisms were criticized due to their methodological and technical errors. However, these studies opened a new route for researchers in terms of the search of methods of cholesterol level reduction in the human organism, increasing the chances of the modern human populations in the combat with cardiovascular disorders [7–13].

The interpretation of study results concerning the influence of lactic acid bacteria on the cholesterol level obtained under in vivo conditions on living organisms is not easy. Organisms of animals and humans differ in terms of mechanisms of regulation of lipid metabolism, including cholesterol. It should be taken into account that introduction of lactic acid bacteria to the gastrointestinal tract does not only have a direct influence on the cholesterol metabolism but also on the entire intestinal microflora, which is capable of metabolizing cholesterol and other lipids, as indicated by the study results obtained by Hosono et al. [14]. This might be the cause for the difficulties in proving the positive influence of lactic acid bacteria on the cholesterol level in the human organism.

Certain in vitro studies from this field conducted within the last dozen or so years enabled assumption that it is lactic acid bacteria that produce the effect of cholesterol level decrease in humans and animals consuming fermented milk products. Numerous study results are available in the literature concerning cholesterol level reduction under laboratory conditions in model media. Decrease of the cholesterol level in culture media has been determined for numerous species and strains of lactic acid bacteria. The majority of research concerns thermophilic bacilli of the genus *Lactobacillus* [14–22]. Other genera of bacteria exhibiting similar property include *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Leuconostoc* [20, 21, 23–26]. According to these studies, the cholesterol binding capacity can be exhibited not only by strains with probiotic characters documented by research but also certain lactic acid bacteria species that are traditionally used to manufacture dairy products and included in dairy starter cultures.

It should be borne in mind that despite the results of in vitro and in vivo studies on animals and humans, it is impossible to unambiguously confirm or negate the capacity of lactic acid bacteria to reduce the cholesterol level in the blood serum due to the possibility of methodological and technical errors and the lack of reproducibility [27]. The more so that the level of cholesterol in blood serum is positively correlated not only with the amount of cholesterol taken with food but also depends on the intake of saturated fatty acids and refined carbohydrates. Therefore, the definite confirmation of the manner in which lactic acid bacteria exercise a beneficial influence on the level of cholesterol in humans is still missing [28–30].

3. Mechanism of cholesterol level reduction by lactic acid bacteria including *Lactococcus* in humans

The assumption that lactic acid bacteria may cause reduction of the cholesterol level directly in fermented milk products or live organisms was made on the basis of numerous in vivo and in vitro studies demonstrating that certain lactic acid bacteria produced a reduction of the cholesterol level in the blood serum of experimental animals or human volunteers or in model culture media. This type of research has been conducted since the 1970s [5, 7, 8, 14, 31–36]. The majority of these studies concern the influence of consumption of fermented products or products containing lactic acid bacteria strains, including primarily probiotic strains. In that time, several scientific hypotheses were formed on the mechanisms through which the phenomenon of cholesterol level reduction performed by lactic acid bacteria may occur. Literature data lists here primarily cholesterol binding, enzymatic deconjugation of bile salts, production of exopolysaccharides, and synthesis of short-chain fatty acids (SCFAs) [15, 21, 23–25, 28, 29, 36–45].

Cholesterol binding by the bacterial cell wall and its incorporation into the cell wall or cytoplasmic membrane of bacterial cells are listed among the major mechanisms [9, 19, 23–25, 43]. It is known that cholesterol binding may have different paths. Certain bacteria incorporate cholesterol into the cell wall, as exhibited by

such genera as *Micrococcus*, *Bacillus*, *Proteus*, or *Mycoplasma*. In the case of lactic acid bacteria, it has been thus far believed that cholesterol is solely attached by the cell through physical adhesion and it is not subject to subsequent metabolism. However, in vitro tests demonstrated that lactic acid bacteria are also capable of incorporating cholesterol into the cell wall [19, 23, 28, 30]. Many scientists have determined that the amount of cholesterol bound by lactic acid bacteria cells depends on, among others, genus, species, and culture of bacteria, growth phase, viability, and cell count [5, 10, 23–25, 38–40, 43]. Research results demonstrate that the strains commercially used in fermented food production are less efficient in binding cholesterol in comparison with the strains isolated from the alimentary tract of humans and animals [16, 46]. According to the majority of literature data, the phenomenon of cholesterol binding by lactic acid bacteria occurs primarily in anaerobic conditions and with the presence of bile salts [5, 17, 35, 47]. However, scientific reports have been published indicating a lack of or poor correlation between tolerance of bile salts and the capacity to bind cholesterol [22, 46, 48].

Another proposed mechanism for cholesterol level reduction in the human organism by lactic acid bacteria is the deconjugation of bile salts, associated with the activity of bile salt hydrolase (BSH) enzyme [16, 17, 21, 30, 39, 40, 45, 49, 50]. Bile salt hydrolase also referred to as cholylglycine hydrolase EC.3.5.1.24 catalyzes hydrolysis (also known as deconjugation) of the amide bond in bile acids conjugated with taurine or glycine, with the release of primary bile acids and amino acids, taurine or glycine [30, 45]. Hydrolysis of bile salts conjugated with taurine or glycine is one of the best known microbiological biotransformations of bile salts. BSH activity is observed for certain bacteria species isolated from the alimentary tract of humans and animals, i.e., strains from genera *Lactobacillus*, *Enterococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides*, that is, microflora from environments rich in conjugated bile acids [14, 30, 35, 39, 40, 45, 51, 52]. A study conducted by Tanaka et al. [53] demonstrated BSH activity also in *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *S. thermophilus* strains. The details on the function of BSH are unknown. It is believed that a relationship exists between BSH activity and the natural environment of bacteria. It is likely that hydrolysis of bile salts catalyzed by BSH constitutes a protection mechanism against the toxic effect of these salts, present in the natural environment of the bacteria. As demonstrated in the literature data, the influence of bile salts on the surface of bacterial cells may result in changes in the metabolism and structure of the cell wall and membrane [23, 54–56]. Such changes have been observed in *Lactobacillus* bacteria, among others. However, some researchers believe that bile acids released by BSH are even more toxic toward bacterial cells than their forms conjugated with taurine or glycine [48, 53, 57–61]. Recently, a mechanism has been proposed, according to which BSH facilitates incorporation of cholesterol or bile salts into bacterial cell membrane [62]. The positive effects stemming from the capacity of lactic acid bacteria for bile salt hydrolysis are sometimes understated in the literature. It is believed that deconjugated bile salts may return to the liver and then to the intestines, where the intestinal microflora transforms them into secondary bile salts (SBS), which are considered cytotoxic [63]. Deoxycholate and lithocholate are examples of such secondary bile salts and are formed by removing the 7α-hydroxyl group from primary bile salts, cholane and chenodeoxycholate, respectively [28, 30, 64, 65]. Removal of the 7α-hydroxyl group from primary bile salts is catalyzed by an enzyme known as 7α-dehydroxylase. It is suspected that BSH along with 7α-dehydroxylase plays a significant role in the gallstone formation [53]. However, no 7α-dehydroxylase activity could be found for *Lactobacillus* strains isolated from humans or dairy products [64, 65]. This debunks the myth that lactic acid bacteria and bifidobacteria contribute to the formation of secondary bile acids and gallstones.

Another probable mechanism of cholesterol level reduction in the blood serum is associated with the capacity of numerous lactic acid bacteria to synthesize exopolysaccharides. However, this mechanism remains among the group of hypotheses that have been poorly understood and studied [28, 29, 42, 66, 67]. It is suspected that EPS influence the absorption of cholesterol, free bile acids, or salts from the intestines through binding and removing them from the organism via the same principle as it is performed by nutritional fiber or plant polysaccharides [42, 68]. Nakajima et al. [67] demonstrated that the level of cholesterol in the blood serum was lowest in the rats fed with milk containing EPS-producing streptococci. Similarly, the HDL cholesterol fraction ratio to its total content was highest in the rats fed with diet including these streptococci. This shows that EPS produced by *Lactococcus lactis* subsp. *cremoris* SBT 0495 had a positive impact on the metabolism of cholesterol in rats. Moreover, results of in vitro tests carried out by Pigeon et al. [42] suggested that bile acid binding by EPS could influence reduction of the cholesterol level via its usage in the synthesis of new bile acids in the place of those associated with EPS and thus removed from the system. Moreover, they formed a hypothesis that the full EPS efficiency in terms of cholesterol or bile acid removal requires the activity of BSH-type enzymes in lactic acid bacteria and bifidobacteria. However, these researchers did not verify whether this relationship is also present with regard to conjugated bile acids (e.g., glycocholic acid, taurocholic acid), as then it would be possible that the phenomenon of bile salt binding by EPS does not require activity of bile salt hydrolase and it may occur in the conditions prevailing in the intestine. Perhaps the cholesterol removal by EPS-producing bacteria is even more complex than in the case of bacteria that do not produce these substances. Moreover, it is unknown whether the cholesterol bound by EPS is biologically available to human organism, as the literature lacks information as to whether this research has been conducted in vivo.

Another mechanism, associated with production of short-chain fatty acids, has been mentioned among other possible mechanisms of cholesterol level reduction in the human organism by lactic acid bacteria [28, 29, 36, 69]. In the human organism, propionic acid penetrates to the liver and inhibits the hypercholesterolemic effect of acetate, the precursor of cholesterol and a product of fermentation activity of lactic acid bacteria. Thus far no in vivo tests have been conducted to confirm this phenomenon. St-Onge et al. [36] further point out to the fact that synthesis of acetate by lactic acid bacteria predominates synthesis of other SCFAs.

The aforementioned supposed mechanisms concern reduction of the level of cholesterol in the blood serum by lactic acid bacteria. It is presumed that considering lactic acid bacteria and bifidobacteria do not metabolize cholesterol, then it is possible that only binding (adhesion and/or assimilation) of cholesterol by cell wall or cytoplasmic membrane occurs in food products. Thus far, it has not been demonstrated that lactic acid bacteria are capable of metabolizing cholesterol, although the literature provides examples of studies on the introduction of genes encoding activity of such genes to the cells of lactic acid bacteria [70–73]. It is known that many other microorganisms produce enzymes that decompose cholesterol to other compounds, e.g., cholesterol reductase or cholesterol oxidase [70, 71, 74, 75]. Worth noting are intestinal microorganisms producing the enzyme of cholesterol reductase that transforms cholesterol into coprostanol (5β-cholestan-3β-ol). In the human organism, the anaerobic intestinal microflora transforms cholesterol primarily to 5β-coprostanol [76]. It should be noted that coprostanol is poorly absorbed in the gastrointestinal tract and it is easily eliminated from the organism [14]. *Eubacterium coprostanoligenes* is a bacteria species that includes cholesterol reductase. These bacteria could be used for production of probiotic foods with a naturally reduced cholesterol level, and such attempts have been made, yet thus far

with poor effects [77]. In vivo tests on animals demonstrated that administration of *Eubacterium coprostanoligenes* has a positive influence on reduction of cholesterol in the blood serum [29, 78–80]. This is an indication that providing lactic acid bacteria cells with the capacity for cholesterol transformation into coprostanol may enable reduction of the cholesterol level already at the stage of fermented product formation.

By examining the hypocholesterolemic influence of lactic acid bacteria on the level of cholesterol in the blood serum of volunteering humans or experimental animals, it should be borne in mind that introduction of additional microflora to the intestines may significantly alter the quantitative and qualitative composition of the entire intestine ecosystem and its function. As shown in the results of the study of Hosono et al. [14], despite the fact that lactic acid bacteria cells do not possess the capacity to transform cholesterol into coprostanol, they are capable of influencing the amount at which it is excreted from the organism. This forms the effect of the influence of lactic acid bacteria on the remaining microorganisms present in the intestinal microflora.

4. Cholesterol binding sites by bacterial cells

As stated above, binding (adhesion or assimilation) by bacteria cells is one of the major mechanisms for the removal of cholesterol by bacteria from the environment. Hosono and Tono-Oka [24] have suggested that it is the chemical nature and structure of peptidoglycan present in bacterial cell wall that fulfill a major role in cholesterol binding. This hypothesis was confirmed by Usman and Hosono [43]. They further suggested that a portion of cholesterol could be embedded into the bacterial cell walls. The possibility for incorporating cholesterol into the cell membrane of lactic acid bacteria was demonstrated in the study of Noh et al. [19].

The phenomenon of cholesterol binding by the cell wall has been indicated by similar research conducted on the binding of aflatoxin B1 (AFB1) by lactic acid bacteria cells [81–86]. Many researchers point out to the phenomenon of AFB1 aflatoxin binding by live and dead lactic acid bacteria cultures, which do not possess the capacity to metabolize this compound [81, 83–85, 87, 88].

As it is known, Gram-positive bacteria are characterized by a thick cell wall. The cell wall of Gram-positive bacteria comprises of peptidoglycan (murein) and its associated teichoic and/or teichuronic and lipoteichoic acids and proteins [89]. The wall thickness ranges between 15 and 50 nm, corresponding to 20–30 individual murein layers. Murein is built of saccharide chains comprising of alternately arranged N-acetylglucosamine and N-acetylmuramic acid, joined with a β-(1 → 4)-glycoside bond [89]. Apart from the saccharide chains, murein contains short peptides. The free carbonyl group of muramic acid forms the acceptor for the first peptides amino acid. Typically, L-alanine is the first amino acid. The protein portion of murein exhibits considerably greater diversity than its saccharide part, as its composition depends on the bacteria species, environmental conditions, and even the cell age. In Gram-positive bacteria, the cell wall further contains proteins active in various physiological and biochemical processes—energy transfer, electron and proton transport, cell casing synthesis, etc. [89]. Moreover, various types of polymers are associated with murein, such as teichoic acids (teichoic and lipoteichoic acid) and teichuronic acid. Considering the manner in which these acids are attached, they are sometimes referred to as secondary (after murein) polymers of bacterial cell wall. The importance of these acids has not been fully explained, although numerous assumptions have been made explaining the presence of these compounds in the bacterial cell wall. It is possible that these acids play a certain

role in bacteria adhesion, biofilm formation, tolerance to environmental acidity, resistance to antibiotics, bacteriophages, or UV radiation. Lactic bacteria synthesize teichoic and lipoteichoic acids simultaneously or lipoteichoic acids only. The characteristic feature of lipoteichoic acids is the presence (at the end of the chain) of a glycolipid anchored into the cytoplasmic membrane, and the structure of this connection depends on bacteria species. The qualitative composition of cytoplasmic membrane phospholipids depends on environmental factors, such as availability of nutrients, temperature, pH, and presence of toxic materials. The fatty acids profile changes also depending on the genus and species of bacteria and their growth phase, which has been used for microorganism grouping and classification attempts [69, 74, 90]. C16 fatty acids are the most common, while C12, C14, and C18 fatty acids are less frequently found. Methylated, hydroxylated, and branched fatty acids or those containing cyclopropane ring are common. Lactobacillic acid—a fatty acid containing cyclopropane ring—was first detected in the cytoplasmic membrane of lactic bacilli [50, 69].

Literature data indicate that the cell wall or cytoplasmic membrane can form the cholesterol binding site. In the case of the cell wall, the bond may have physical (adhesion) or chemical character (analogous to incorporation of teichoic, lipoteichoic, and teichuronic acid incorporation). In the case of a chemical bond, we deal with cholesterol assimilation, that is, its incorporation into the cell wall. Cholesterol molecules are oriented in the cell membrane in the same manner as phospholipid molecules. The polar portion of cholesterol molecule adheres to the polar portion of phospholipid. Perhaps, in cytoplasmic membranes of bacterial cells, cholesterol molecules are located in the same manner as in membranes of eukaryotic organisms. The bacterial cytoplasmic membrane contains compounds with a structure similar to steroids, which further indicates the possibility for cholesterol incorporation into the cytoplasmic membrane of bacterial cells. However, in order to be incorporated into the cytoplasmic membrane, cholesterol molecules must be transported through the cell wall. As shown in the studies of Kurdi et al. [91], Pigeon et al. [42], and Kurdi et al. [61] on bile acids, transport of such large molecules through the cell wall is possible even if it threatens the survival of the bacteria. Cholesterol binding by the cell membrane is not neutral to the bacterial cell itself. The presence of such substances as cholesterol in the environment influences the ratio of saturated acids to unsaturated acids in the cytoplasmic wall, as well as the structure and properties of this membrane. Goldberg and Eschar [92] noted that addition of Tween 80 to culture medium increases the concentration of certain fatty acids with the concomitant influence on the ratio of saturated acids to unsaturated acids. The same happens when the cholesterol molecules are being bound. Dambekodi and Gilliland [23] proved that incorporation of cholesterol into the cell membrane of bifidobacteria was manifested by changes in its composition and resulted in an increase of the resistance of cells growing in the presence of cholesterol to ultrasonic lysis. In turn, Taranto et al. [50] demonstrated that bacterial cells growing in the presence of cholesterol or bile salts are more resistant to lysis than those growing in their absence, contrary to the cells growing in the absence of cholesterol. The cited authors observed that addition of cholesterol to culture broth resulted in an increase of saturated fatty acid content in lactic bacilli biomass from 44.3% to 56.5% of total acids and unsaturated acids from 1.26% to 43.5% of the total amount of fatty acids. Furthermore, Kimoto et al. [25] reached a conclusion that the change in the distribution of fatty acids by *Lactococcus lactis* cells growing in the presence of cholesterol is an effect of its removal from the culture medium and incorporation into the cell membrane. Liong and Shah [38] examined the influence of cholesterol on the profile of fatty acids of lactic bacilli and determined that the strains growing in the medium without addition of cholesterol demonstrated a stronger percentage content of

unsaturated acids (oleic and linoleic acids) than the samples, to which cholesterol was added. According to Boggs [93] cholesterol forms hydrogen bonds with the amide group N—H of bile acids and oxygen molecules of hydroxyl groups of saccharides in fatty acids. It is likely that the same bonds connect the cholesterol with phospholipids and glycolipids of bacterial cell membrane [50]. However, according to other literature data, no strict relationship exists between lactic acid bacteria resistance to bile salts and their capacity to bind cholesterol [46, 48].

5. Influence of selected factors on cholesterol removal by *Lactococcus* cells

It can be stated that the phenomenon of cholesterol binding and removal by bacterial cells is complex. It can be concluded that the contribution of the phenomenon of cholesterol molecule assimilation or adhesion by lactic acid bacteria cells depends on a wide range of factors, which are not always possible to reproduce or replicate in subsequent experiments. Perhaps this depends on the different chemical structure of the cell wall, particularly peptidoglycan, as well as lipid profile of phospholipids of the cytoplasmic membrane in bacterial cells.

5.1 Influence of cholesterol concentration on cholesterol removal by *Lactococcus*

The capacity of lactic streptococci to reduce the cholesterol level under in vitro conditions was also tested by Hosono and Tono-Oka [24] and Kimoto et al. [25]. The cited researchers carried out cultures at 37°C for 24 h. In the study of Hosono and Tono-Oka [24], the percentage of cholesterol bound by *Lactococcus lactis* subsp. *lactis* 12007 and 12546 strains was 25.1 and 30.3%, respectively. Four strains of *Lactococcus lactis* subsp. *cremoris* bound from 14.2 to 20.9% of cholesterol and two strains of *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis*—29.7 and 33.9%, respectively. The capacity to remove cholesterol from culture broth was demonstrated also in the case of *Leuconostoc mesenteroides* subsp. *cremoris*, and it amounted to between 11.4% and 14.9%, depending on the strain. In turn, in the experiments of Kimoto et al. [25], bacterial cells from *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* strains removed 53.9–86.7% and 31.0–97.3% of cholesterol, respectively, from GM17-THIO broth, containing addition of 0.2% sodium taurocholate and 0.070 g cholesterol per 1 dm^3 of medium. Moreover, Ziarno [4] examined the capacity of isolates from the genus *Lactococcus* originating from fermented dairy products to remove cholesterol depending on the concentration of cholesterol in culture broth (in a range from slightly above 0 g/dm^3 to close to 2 g/dm^3). Considering it is known that lactic acid bacteria do not metabolize cholesterol, its loss from post-culture liquid can be seen as the amount of cholesterol removed and bound by bacterial cells. Ziarno [4] demonstrated that the amount of cholesterol removed by bacterial cells is determined by the preliminary concentration of this substance in the culture medium. In general, the more cholesterol was introduced to the culture broth, the more of it was removed by bacterial cells. However, the above statement is true only for low cholesterol concentrations in culture broth. With higher concentration of cholesterol in the culture broth, amounting to over 1–1.5 g/dm^3, its removal by bacterial cells was still observed; however the dynamics of this removal was far less pronounced than in broths with lower cholesterol concentration. The earlier research indicates a different capacity of lactic acid bacteria cultures to remove cholesterol from culture media [6, 94, 95]. The differences were observed between individually tested cultures and between individual replications for the same culture. This is a confirmation of observations

of other researchers [5, 6, 10, 16, 17, 21–26, 38, 41, 46, 48, 96]. A significant effect on the diversity of the results obtained not only within the strains but also repetitions seems to be also held by the fact that the mechanism of cholesterol binding by bacterial cells can occur via adhesion of cholesterol molecules through the cell wall or by embedding it into the cell wall or membrane [9, 19, 23–25, 43]. It appears to be obvious that cholesterol adhesion does not produce strong binding, and this substance is very easily washed back to the culture broth. In turn, embedding cholesterol into the cell wall or cytoplasmic membrane is more durable. This may explain the observed considerable dispersion of results and the lack of experiment reproducibility.

5.2 Influence of culture temperature on cholesterol removal by *Lactococcus*

Usman and Hosono [43] demonstrated that *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* bacteria are capable of binding and removing cholesterol already after culture is started, independent of its temperature in the range from 10 to 70°C. After addition of salts of such metals as Mg^{2+}, Na^{+}, Ca^{2+}, or K^{+}, the cholesterol binding was inhibited. The bacteria bound the highest amount of cholesterol when the pH value was about 7.0. The applied culture temperature range indicates that dead bacterial cells are also capable of binding cholesterol, which comprised the subject of further tests of this study. In turn, Noh et al. [19] demonstrated that lactic bacilli bind cholesterol in a culture with constant pH of 6.0, as well as during growth without pH value control. Ziarno [4] examined the capacity of *Lactococcus* cells isolated from industrial dairy starters to remove cholesterol in M17 culture broth with application of several temperature variants of culture (4, 25, and 30°C). The temperature of 4°C aimed at stimulating refrigeration conditions and ensuring inhibition of bioactivity of bacterial cells [97, 98]. The temperature of 30°C was utilized as the optimum conditions for the development of mesophilic bacteria. In turn, the temperature of 25°C was used to simulate room temperature conditions. It was proven that bacterial cells from all tested lactic acid bacteria cultures reduced the level of cholesterol in culture medium in the applied experimental conditions. As it could be expected, the degree at which cholesterol is removed depended on the applied temperature of lactic acid bacteria incubation. The initial cholesterol concentration in culture broths was on average 0.606 g/dm^3. When the cultures were kept at the temperature of 4°C, mesophilic cultures of *Lactococcus* removed low amounts of cholesterol (from 0.005 to 0.021 g/dm^3) [4]. When the cultures were carried out at 25°C, the discussed cultures bound from 0.065 to 0.085 g/dm^3. In turn, at the temperature of 30°C, which is optimum for the development of mesophilic cultures, the obtained values of removed cholesterol ranged from 0.068 to 0.104 g/dm^3 [4].

5.3 Influence of *Lactococcus* live cell biomass concentration on cholesterol removal

Usman and Hosono [43] determined that cholesterol binding was significantly dependent on the amount of bacterial cell biomass and it increased proportionate to the increase of the cell count. Furthermore, Liong and Shah [38] observed that the amount of cells has a significant impact on the differences in the amount of cholesterol bound by lactic acid bacteria, whereas the growth dynamics for individual strains determines the amount of cell biomass and differences in experimental results. Ziarno [4] verified the manner in which the concentration level of live cell biomass originating from monocultures and multi-species cultures of *Lactococcus* influences the capacity of cultures to remove cholesterol from M17 culture broth. As expected, the highest amount of cholesterol was removed in the cultures containing 10-fold concentrated *Lactococcus* biomass. At this cell biomass concentration, the

studied *Lactococcus* cultures removed on average between 0.113 and 0.129 g/dm^3 of cholesterol from its initial content of 0.611 g in 1 dm^3 of M17 broth. In turn, bacterial biomasses with a 10-fold lower concentration (1×) produced approximately 1.3–1.6-fold reduction of the amount of cholesterol removed. From a culture broth containing a 10-fold diluted bacterial cell biomass culture, from 0.054 g/dm^3 to 0.066 g/dm^3 of cholesterol was removed after culture maintained for 20h, thus 1.3–1.5 times less than in the case of 1× concentrated biomasses [4]. It is worthy of emphasis that in multi-species commercial mesophilic starter cultures, used in the dairy industry, e.g., cheese and cream production, similar capacities to remove cholesterol were observed as in lactic acid bacteria monocultures. However, it should be expected that with 10-fold decrease of cell biomass concentration, the amount of cholesterol removed from culture broth will be decreased proportionately (by 10-fold). However, minor differences were observed in the amount of cholesterol removed by biomasses with selected live cell concentration levels. This can be explained with two phenomena. Firstly, the applied cultures were live and biologically active. During the experiments, bacteria propagated, significantly altering the amount of biomass capable of binding cholesterol. Microbial analyses demonstrated that the strongest increase of *Lactococcus* population was observed in the culture with the lowest initial biomass concentration (10-fold diluted). Bacteria propagation was poorest in the cultures with the highest initial concentration of biomass (10×). After completion of experiments, in the cultures containing 10-fold diluted biomass of the tested mesophilic cultures, the live cell bacteria count was determined at 7–8 log CFU/cm^3. In cultures with 10-fold concentrated biomass, an average of 6–7 log CFU/cm^3 was determined [4]. The second explanation for the minor differences in the amount of cholesterol removed by *Lactococcus* biomass with the used live cell concentration levels is the concomitant adhesion and assimilation of cholesterol molecules. Most likely, with poor growth of bacterial cells, the phenomenon of cholesterol removal through its adhesion by the cell wall is predominant. And as it could be expected, this type of cholesterol binding is not durable and cholesterol is easily released. In turn, the high biological activity of bacterial cells may favor permanent embedding of cholesterol into the wall or cytoplasmic membrane of bacteria cells, which likely occurred in the experiments of this stage of research, in cultures with the lowest initial biomass concentration (diluted 10-fold), in which the greatest increase in population was observed.

The obtained study results may find implications for the explanation of hypocholesterolemic influence of products containing lactic acid bacteria. A considerable amount of literature data is available on the subject, but these are often contradictory [5–7, 10–13]. Based on the results of this study, a hypothesis can be formed that in this case the count of live bacteria in the product is important. In order for bacterial cells to assimilate cholesterol molecules, their high biological activity is required, as demonstrated by Hosono and Tono-Oka [24] for *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* R-43 strain; the course of this phenomenon is most intensive in the logarithmic growth phase. The physical binding of cholesterol by the cell wall does not require cell activity, only a suitably long contact time between the cells and cholesterol molecules. The same team of researchers noted that not only live but also dead cells of the tested strain were capable of binding cholesterol.

5.4 Influence of *Lactococcus* dead cell biomass concentration on cholesterol removal

The sparse literature data on cholesterol removal by inactivated cells prove that lactic acid bacteria monocultures are capable of removing cholesterol from culture media even after their thermal death [21, 24, 25, 43, 95]. The amount of cholesterol

removed by inactivated cells is considerably lower than by biologically active cells, which likely stems from the fact that in the case of dead cells cholesterol may not be built into the cell wall or cytoplasmic membrane, but it only undergoes adhesion by the cells. Furthermore, Ziarno [4] demonstrated that biomass of dead (thermally inactivated) cells of *Lactococcus*, originating from industrial monocultures and multi-species cultures, influences cholesterol uptake from the M17 culture broth. The highest amount of cholesterol was removed in the cultures containing 10x concentrated biomass of dead bacterial cells, from 0.074 to 0.083 g/dm^3 of cholesterol from the M17 broth. Bacterial biomasses with 1x concentration removed 1.4–1.9-fold less cholesterol. Tenfold diluted biomass of dead bacterial cells bound from 0.021 to 0.029 g/dm^3 of cholesterol, thus 1.7–2.3-fold less than 1× concentrated biomasses. The fact that cholesterol removal occurs even when the bacterial cells are dead confirms that the physical binding of cholesterol molecules by the cell wall (adhesion) is one of the mechanisms of cholesterol removal by *Lactococcus* cells.

6. Survival of *Lactococcus* cells in the human gastrointestinal tract

Literature contains studies confirming the capacity of lactic bacilli to survive under in vivo conditions in the human alimentary tract [28, 30, 35, 52, 99, 100]. The factors with a significant impact on lactic acid bacteria survivability in the alimentary tract include low gastric pH value, intestine peristalsis, presence of bile acids in the pancreatic fluid and various digestive enzymes present in the individual sections of the alimentary tract, presence of nutrients, as well as bacteria passage time through the alimentary tract and their initial count [35, 50, 101–103]. The mentioned factors result in a decrease of lactic acid bacteria survival rate, but at the same time they may constitute a criterion for the selection of probiotic strains [28, 104].

In order to determine the survival rate of lactic acid bacteria, scientists first determine their resistance to low pH present in certain sections of the gastrointestinal tract. Gastric fluid comprises of the secretion of foveolar cells secreting mucus, chief cells secreting digestive enzymes (pepsin), and parietal cells secreting hydrochloric acid. The pH of gastric fluid is between 1.5 and 3.0. Secretion of gastric fluid is inhibited when the pH drops below 2.0. The temperature inside the stomach is over 37°C, and the alimentary content, depending on the individual physiological and emotional circumstances, remains in the stomach for average 1–3 h [4]. Results of in vitro tests concern survival rate of different lactic acid bacteria strains under conditions imitating low pH of the gastric fluid [18, 35, 41, 105–109]. Strains traditionally used to manufacture dairy products have also been commonly found to survive the conditions of gastric fluid [101]. Also the study of Lankaputhra and Shah [107] indicated that numerous lactic bacilli strains survived perfectly the conditions simulating the pH of gastric fluid.

Another subject of the study is the capacity of lactic acid bacteria to survive during transport through subsequent sections of the alimentary canal. Here, a particular significance is exhibited by the section of the small intestine [102, 110, 111]. Literature data show that bile salts comprise a serious obstacle for lactic acid bacteria, as they contain toxic bile acids [19, 106, 107, 112]. Ziarno and Bartosz [113] provided evidence for the influence of cell biomass on the survival of lactic bacilli in model intestinal fluid. Cholesterol influences the composition and functioning of the bacterial cell wall and membrane, thus producing change in the relationship with the surrounding environment, such as resistance to bile acids, pH, or temperature [46, 48, 50]. Cholesterol uptake by bacterial cells is not neutral to them and results in a change of, among others, the profile of fatty acids of the cell membrane [23, 25, 38, 50]. Cell responds to stress conditions of the environment with a change of the composition of the cell membrane, and it may result in an increase of the

resistance of the cell to stress factors [50]. Doubtlessly, this is significant for the survival of lactic acid bacteria in various environments they inhabit, such as the alimentary tract or food products.

The strains which are not probiotics exhibit lower survival rate of their cells in model gastric fluid as compared with probiotic strains [41, 108]. Ziarno and Margol [109] examined the capacity of bacteria from several mesophilic starter cultures to survive in a simulated gastric fluid. Also in their study, industrial starter cultures containing bacteria from the genus *Lactococcus* were used, which, after propagation, were kept in a simulated gastric fluid with pH of 2.4 for 3 h at 37°C. The study demonstrated that the present streptococci were not resistant to the environment of a simulated gastric fluid [109]. On the other hand, intestinal fluid has a more complex enzymatic and chemical composition than broths used by other researchers, but its influence on lactic acid bacteria cells is typically referred to probiotic strains of thermophilic lactic acid bacteria [38–40, 108, 110, 111]. Ziarno [4] tested the viability of *Lactococcus* in model conditions of the alimentary tract in the presence of cholesterol, separately for the simulated gastric fluid and simulated intestinal fluid. *Lactococcus* isolated from industrial starter cultures were used for the experiments. No influence of addition of cholesterol on the viability of *Lactococcus* cultures in a simulated gastric fluid could be demonstrated, although reduction of live cells in the range from 1 to 3 log CFU/cm^3 was observed. *Lactococcus* cells exhibited low tolerance also to the conditions of simulated intestinal fluid, considerably lower than the simulated gastric fluid. From the initial cell population of average 6–7 log CFU/cm^3, only 2–3 log CFU/cm^3 remained after 6 h of experiment, with few exceptions surviving at the level of 6 log CFU/cm^3, independent of the addition of cholesterol. The lack of influence of cholesterol in simulated intestinal fluid on the survival rate of lactic acid bacteria cells was also demonstrated in earlier research [114, 115]. The study conducted by Ziarno [114, 115] utilized bacteria cultures isolated from commercial pharmaceutical preparations and commercially available dairy products or dairy starter monocultures. Cells of lactobacilli tolerated conditions of simulated intestinal fluid better than bifidobacteria cells and *Lactococcus lactis* cells.

The good tolerance of bacterial cells to the conditions of simulated digestive fluids can be explained by the occurrence of these bacteria in the alimentary tract of humans and animals. Numerous factors determine lactic acid bacteria viability, including pH, temperature, oxygenation, and presence of toxic substances toward bacterial cells [101–103]. Bacteria not forming the natural intestinal microflora do not possess the natural resistance to the conditions of the intestinal fluid [106]. Viability of bacterial cells determines the level of cholesterol removal. It seems obvious that the count of live and dead bacterial cells holds significance for the removal of cholesterol under the conditions of a human alimentary tract. Thus, a hypothesis can be formed that the factors determining survival rate of bacterial cells further influence the cholesterol removal level by lactic acid bacteria and bifidobacteria cells. Such relationships may further impede interpretation of the results of experiments realized under in vitro or in vivo conditions and may prevent interpolation of results obtained in vitro onto the conditions of human or animal organisms.

7. Cholesterol uptake and release by *Lactococcus* in the simulated human gastrointestinal tract

7.1 Cholesterol uptake by *Lactococcus* under conditions of simulated gastric fluid

Cholesterol uptake by *Lactococcus* cells in simulated gastric fluid depends on the amount of biomass [4]. Ziarno [4] carried out in vitro experiments with the use of

industrial starter cultures of mesophilic lactic bacteria, including *Lactococcus*. The cultures were grown for 5 h at 37°C in a simulated gastric fluid containing addition of 0.511 g/dm^3 of cholesterol. The study demonstrated that higher amount of cholesterol was bound by *Lactococcus* cells contained in mixed cultures than cells from *Lactococcus lactis* monocultures. Bacterial cells present in the mixed cultures removed cholesterol in the range from 0.012 to 0.020 g/dm^3. In turn, bacterial cells from *Lactococcus lactis* cultures reduced cholesterol concentration in the simulated gastric fluid by an average 0.005 g/dm^3. 10× concentrated bacterial biomasses removed 1.4–2.3 times more cholesterol than bacterial cells with 1× cell concentration. In turn, biomasses with 0.1× bacterial cell concentration bound 2.2–4.6 less cholesterol than bacterial cultures with 1× cell concentration. This means that the conditions prevailing in the stomach may favor removal of cholesterol by bacterial cells independent of their viability. However, it remains unknown whether bacterial cells release the bound cholesterol after entering the gastrointestinal tract and whether it may penetrate to the blood. Similar studies concerning aflatoxin B bound by the cell wall of lactic bacilli suggest that such assimilation by the cell wall may be robust [81–84]. This may indicate that cholesterol binding is also robust.

7.2 Release of cholesterol bound by *Lactococcus* in the conditions of simulated gastric fluid

The study of Ziarno [4] indicates that the binding of a portion of cholesterol by lactic acid bacteria cells is robust enough so that it is not released in the conditions of gastric fluid. The study was carried out using isolates of *Lactococcus* originating from industrial monocultures and mixed cultures. Bacterial cells present in the tested cultures released 51–84% of the removed and bound cholesterol independent of bacterial cells' viability. The biomass of dead cells released lower amount of cholesterol than the biomass with viable cells, but it also bound and removed lower amount of cholesterol from the culture medium earlier. Biomass of live *Lactococcus lactis* cells removed an average of 0.063 g cholesterol/dm^3, whereas biomass of dead cells removed average of 0.033 g/dm^3 [4].

Similar tendencies are observed in the case of studies conducted on aflatoxin B1 binding by lactic acid bacteria [81, 84]. El-Nezami et al. [81] observed that aflatoxin B1 uptake from culture medium by selected lactic acid bacteria cultures depended on their population and culture temperature. The same was demonstrated by Lee et al. [84]. Identical relationships were observed in the present study with regard to binding and release of cholesterol by lactic acid bacteria cells. Moreover, Lee et al. [84] concluded that thermal killing of bacteria resulted in a change of the surface of bacteria cells and uncovering of additional binding sites for aflatoxin B1.

7.3 Cholesterol removal by *Lactococcus* in the conditions of simulated intestinal fluid

As stated by Ziarno and Bartosz [113], cholesterol removal by lactic acid bacteria in intestinal fluid is less pronounced than in culture broth. This is further confirmed by the experiments of Ziarno [4] conducted under in vitro conditions with *Lactococcus* isolates originating from industrial starter cultures. The mentioned cultures were grown at 37°C for 6 h in a simulated intestinal fluid with addition of cholesterol. The tested *Lactococcus* cultures resulted in a reduction of cholesterol from the initial content of 0.543 g/dm^3 to the level between 0.011 and 0.087 g/dm^3. In the majority of the tested cultures, the influence of biomass concentration on the degree of cholesterol removal was statistically significant; however, 10-fold concentrated biomass did not remove 10 times more cholesterol than onefold concentrated

biomass. Therefore, Ziarno [4] demonstrated that in not all of the tested *Lactococcus* cultures the degree of biomass concentration had a significant influence on the amount of cholesterol removed. This can be explained by the activity of enzymes such as BSH, which caused bile hydrolysis and coprecipitation of cholesterol with released bile acids, independent of the amount of cells in the culture.

The chemical composition of simulated intestinal fluid seems to be of significance for the obtained results [18]. This indicates additional methodological factors influencing the results obtained in laboratory experiments conducted under in vitro conditions. In order to prepare simulated intestinal fluid, cattle bile was also used containing conjugated and deconjugated bile salts; therefore bile salt hydrolase activity (produced by the majority of intestinal lactic acid bacteria strains) was not necessary for cholesterol precipitation with free bile acids to occur [30, 35, 39, 40, 45]. Active BSH enzyme results in hydrolysis of bile salts, whereas cholesterol molecules may coprecipitate with the released bile acids [16, 17, 21, 30, 35, 39, 40, 45, 49, 50]. Such phenomenon has been observed in numerous lactic acid bacteria species, but not in *Lactococcus* thus far [49, 53]. It is known that coprecipitates of cholesterol with bile acids are formed at a low pH below 5.5 [17, 18, 21, 39, 49, 52]. However, with a renewed increase of pH to over 5.5, such coprecipitates were rapidly dissolved [15, 18, 28, 39]. Bile secreted from the liver is introduced to the duodenum, where it neutralizes the acidic food pulp that leaves the stomach and then the pH in the small intestine has a value of over 6.0. Under these conditions, the coprecipitates of bile acids and cholesterol are dissolved. Thus, the hypocholesterolemic effect caused by cholesterol coprecipitation with deconjugated bile acids is probably impossible to occur under in vivo conditions.

7.4 Release of cholesterol bound by *Lactococcus* in the conditions of simulated intestinal fluid

Ziarno [4] studied whether the cholesterol previously bound by *Lactococcus* cells is released under the conditions of simulated intestinal fluid. It was determined that certain isolates of *Lactococcus lactis* released up to 60–90% of cholesterol, which was earlier bound by these cells. Lower amount of cholesterol under conditions of simulated intestinal fluid is released by *Lactococcus lactis* cells (average of 45%), meaning that in these bacteria cultures cholesterol was bound with sufficient force by the cell wall so that it was not released under the conditions of simulated intestinal fluid. This may confirm the hypothesis of Lee et al. [84] on structural changes in the wall of dead bacterial cells.

8. Conclusions

One important conclusion should be drawn from the research results presented above, namely, that lactic acid bacteria may cause a different hypocholesterolemic effect in the human digestive system. They may exhibit a clear capacity for permanent binding and removal of cholesterol or to not bind it at all. It is also possible that they may cause such change of the intestinal microflora. Hosono et al. [14] formed a hypothesis that lactic acid bacteria may influence the amount of cholesterol eliminated from the organism despite the fact that they do not have the capacity to transform it into coprostanol. This is an effect of the influence of lactic acid bacteria on other microorganisms present in the intestinal microflora, including microorganisms capable of transforming cholesterol into coprostanol. This is particularly possible in the case of probiotic strains of lactic acid bacteria and bifidobacteria, which are distinguished due to their capacity to produce low-molecular antimicrobial

substances. Based on the results of experiments conducted by Ziarno [4], it can be stated that the phenomenon of cholesterol binding depends on such a wide array of factors influencing the cell wall and cytoplasmic membrane of bacteria that it may not be impossible to predict the hypocholesterolemic effect unambiguously.

It can be concluded that lactic acid bacteria are capable of binding cholesterol molecules present in their environment. Cholesterol can be subject to adhesion by the cell wall or assimilation via the cytoplasmic membrane or cell wall of lactic acid bacteria, including *Lactococcus*. However, the degree and force of this bond depend on numerous environmental factors. A change of even one of these parameters results in the hypocholesterolemic effect which is no longer reproducible in the experiments. It is likely that this is the manner in which the results and the discrepancies found between in vitro and perhaps also in vivo tests on human volunteers and experimental animals should be interpreted and explained.

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Conflict of interest

The author has declared that she does not have any conflict of interest for publishing this research.

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