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Original article

In vitro cholesterol-lowering activity of *Lactobacillus plantarum* and *Lactobacillus paracasei* strains isolated from the Italian Castelmagno PDO cheese

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Abstract – The discovery of several healthy beneficial effects of the consumption of dairy products fermented with some bacterial strains led to the investigation of the functional properties of these microorganisms. One of the most studied properties is the cholesterol-lowering activity of bacteria with probiotic characteristics, mostly isolated from human gut. In this work, eight *Lactobacillus plantarum* and five *Lactobacillus paracasei* strains isolated from cheese were studied in vitro for their cholesterol-lowering action and their acid and bile tolerance. The ability of these strains to remove cholesterol was assessed in de Man, Rogosa and Sharpe (MRS) medium, supplied with cholesterol, and in ultra-high temperature (UHT) whole homogenized milk. Among all tested strains, two *L. plantarum* and three *L. paracasei* strains gave rise to a significant reduction of the cholesterol level in MRS broth; in particular, *L. plantarum* strains lowered the cholesterol content by an average of 19.4%, whereas *L. paracasei* strains lowered by an average of 6.8%. The two *L. plantarum* strains possessing the highest cholesterol-lowering activity in MRS broth were also tested in milk. Results showed that *L. plantarum* strains maintained this activity because after 24 h the cholesterol decrease ranged from about 5.0% to 8.2% without significant variations between the two strains. Results from the binding assay suggested that cholesterol was mainly removed through the adsorption on the cell wall. Data from acid and bile tolerance assays showed that the *L. plantarum* dairy isolates were able to maintain viability at pH 2 and to grow in a medium with bile salts, and therefore were regarded as probiotics or dairy starters for new probiotic or functional food production.

cholesterol removal / dairy culture / bile tolerance / acid tolerance / probiotics

摘要 – 源于意大利 Castelmagno 干酪的植物乳杆菌与副干酪乳杆菌株的体外降胆固醇活性。 研究人员发现使用特定菌株发酵的乳制品能对消费者产生健康有益的影响，于是对这些菌株的功能特性展开了研究。其中研究最多的有分离于人体肠道的益生菌降胆固醇的功能特性。本文研究了从干酪中分离得到的 8 株植物乳杆菌和 5 株副干酪乳杆菌的体外降胆固醇能力和耐胆盐及耐酸能力。分别以添加胆固醇的 MRS 培养基、均质过的全脂 UHT 乳为培养基对待测菌株的降胆固醇能力进行了评价。结果发现：2 株植物乳杆菌和 3 株副干酪乳杆菌在 MRS 中能显著降低胆固醇水平，其中植物乳杆菌降低胆固醇含量平均水平为 19.4%，而植物乳杆菌为 6.8%。然后又将两株具有最高降胆固醇活性的植物乳杆菌在牛乳中进行了测试。结果显示，植物乳杆菌在 24 h 内的降胆固醇活性为 5.0%~8.2%，两菌株之间差异不显著。通过胆固醇结合实验发现胆固醇含量的降低主要是由于细胞壁的吸附

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作用。胆盐与酸的耐受实验显示，植物乳杆菌能够在 pH 2 并添加了胆盐的乳中保持活力，因此该菌株可作为一种新的益生菌用于乳制品的发酵或添加到功能食品中。

降胆固醇 / 乳培养基 / 耐胆盐 / 耐酸 / 益生菌

Résumé – Activité anti-cholestérol de souches laitières de *Lactobacillus plantarum* et de *Lactobacillus paracasei* isolées du Castelmagno, un fromage Italien traditionnel. La découverte de multiples «effets santé» de la consommation de produits laitiers fermentés avec certaines bactéries a incité à étudier les propriétés fonctionnelles de ces micro-organismes. La propriété la plus étudiée est la réduction de cholestérol de la part de ces bactéries, trouvées pour la plupart dans la flore intestinale et pourvues de qualités probiotiques. Dans cette étude, huit souches de *Lactobacillus plantarum* et cinq de *Lactobacillus paracasei*, isolées du fromage, ont été étudiées in vitro pour leur activité anti-cholestérol et leur tolérance à l'acidité et à la bile. La capacité de ces souches à réduire le cholestérol a été testée dans un milieu MRS supplémenté en cholestérol et en lait entier U.H.T. Parmi ces souches, deux *L. plantarum* et trois *L. paracasei* ont entraîné une diminution significative du taux de cholestérol dans le milieu MRS ; en particulier *L. plantarum* a abaissé le contenu en cholestérol de 19,4 % en moyenne, tandis que *L. paracasei* le diminuait de 6,8 %. Les deux souches *L. plantarum* possédant la plus forte activité anti-cholestérol ont aussi été testées dans le lait. Les résultats montrent que les deux *L. plantarum* maintiennent leur activité réductrice étant donné qu'après 24 h le taux de diminution du cholestérol oscille entre 5,0 % et 8,2 % sans variation significative entre ces souches. Les résultats des essais de liaison suggèrent que le cholestérol est principalement refoulé par adsorption sur la paroi bactérienne. Les donnés des tests de tolérance à l'acidité et à la bile indiquent que les souches de *L. plantarum* étudiées sont capables de survivre à pH 2 et de croître dans un milieu chargé en sels biliaires. Par conséquent, ces bactéries peuvent être envisagées comme ferments lactiques pour la production de nouveaux probiotiques ou compléments alimentaires.

activité anti-cholestérol / bactérie lactique / tolérance à l'acidité / tolérance à la bile / probiotique

1. INTRODUCTION

The consumption of food rich in lipids, especially saturated fatty acids and cholesterol, has a predominant role in the rise in heart diseases [9, 10, 23]. Thus, the consumer demand on food with lower amounts of these components and with additional healthy properties is increasing.

Dairy products play an important role in a healthy diet because of their high nutritional value, but since in some cases they also represent a source of lipids, many attempts were carried out to reduce their fat concentration. As the consumption of fermented milk containing bacterial strains provides beneficial effects, such as the reduction of serum cholesterol levels on human health [7, 12, 19], several researches on bacterial strains with potential hypocholesterolemic properties were then carried out. Strains belonging to different species and genera [1, 2, 4, 7, 15, 17, 18, 21], mostly isolated from human gut, have been investigated mainly in vitro regarding their cholesterol-lowering action, and many studies have also been carried out to understand the mechanisms of serum cholesterol reduction. On the one hand, these findings highlighted that bile salt deconjugation, due to bile salt hydrolase, an enzyme active in several lactic acid bacteria (LAB) species, reduced the cholesterol absorption in the intestinal lumen. This occurs by increasing

the demand of cholesterol for de novo synthesis of bile acids or by reducing cholesterol solubility [22]. On the other hand, some authors suggested that some bacteria are able to incorporate into the membrane or adhere to the surface the cholesterol, making it less available for absorption from the intestine to the blood [20]. Most of these experiments were generally carried out in synthetic media enriched with bile salts and in anaerobic condition, to assay the survival of bacteria in the gastrointestinal tract and to determine the ability to reduce cholesterol directly into the intestine. Also some attempts to reduce cholesterol in dairy products, as a valid alternative to the more expensive chemical and physical processes [11, 16], which can lead to the texture alteration and removal of flavours [1], have been performed. Some authors showed, for example, the ability of few human *Lactobacillus* and *Enterococcus* probiotic strains to assimilate cholesterol in butter and cream [1] and the capability of dairy thermophilic starters [14] or kefir cultures [26] to lower cholesterol in milk, but they did not report data on the survival of these dairy cultures at low pH and in the presence of bile salts.

The aim of this work was to explore by an in vitro assessment the ability of lactobacilli, isolated from a typical Italian cheese, to remove cholesterol during growth in synthetic medium and in milk and to evaluate their survival in the presence of bile salts and under acidic condition, to consider them as probiotics or for the production of functional fermented milk and cheeses.

2. MATERIALS AND METHODS

2.1. Strains and culture conditions

Tests were performed with 13, genetically identified [5], lactobacilli strains (eight *Lactobacillus plantarum* and five *Lactobacillus paracasei*) belonging to the culture collection of the Department of Exploitation and Protection of the Agricultural and Forestry Resources, University of Turin (Italy), isolated from Castelmagno, a typical Italian cheese with protected designation of origin (PDO) produced in Piedmont (Northwest Italy) [5]. Castelmagno is a hard and pressed cheese produced in a small scale using traditional practices from raw cow milk and without starter cultures. The ripening time varies from 60 days, at least, to more than 180 days for peculiar productions.

The commercial starter preparation LA-5® (Chr. Hansen, Hørsholm, Denmark), containing *Lactobacillus acidophilus* strain, was used as positive control, as the cholesterol-lowering action of this strain was already documented under experimental conditions similar to those used in this study [14]. Stock cultures were stored in 20% glycerol (Sigma, Milan, Italy) at −20 °C until the experiments started. Working cultures were maintained at 4 °C and were subcultured in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Milan, Italy) before experimental use. Cultures of each strain were taken at the end of the exponential phase of growth, inoculated at 2%, in triplicate, into two different media, MRS broth and ultra-high temperature (UHT) milk (3.55% w/w of fat and 3.20% of protein), and incubated at 37 °C for 24 h. In each test, UHT milk cholesterol content was 0.012% (w/w), whereas MRS broth was supplemented with a stock solution of cholesterol (12% w/v) to obtain a final concentration of 0.012% (w/v). Stock solution was freshly prepared dissolving cholesterol (Sigma, Milan, Italy) in an aqueous solution of 12% Tween 80 (Sigma, Milan, Italy) and 26.4% ethanol (Fluka, Milan, Italy). In each test, the final ethanol concentration was 2.64%.

A UHT whole milk was selected for this trial as it allows to eliminate the interference of indigenous species, and the homogenization process assures the presence of smaller fat globules increasing cholesterol bioavailability towards bacteria. A sterile 8% (w/v)

NaOH (Riedel de Haen, Seelze, Germany) solution was automatically added to the UHT whole milk to maintain the pH value at 6.5 and to avoid coagulation.

2.2. Cholesterol determination

After the incubation time, cells were collected by centrifugation of MRS broth cultures and milk cultures at 1118× *g* for 5 min and at 716× *g* for 1 min, respectively. The supernatants (0.400 mL each) were saponified at 65 °C with 5 mL of 2.8% (w/v) KOH (Fluka, Milan, Italy) methanolic solution. Cholesterol was extracted using a mixture of hexane (Fluka, Milan, Italy) and deionized water (5:1 v/v) [6]. The cholesterol content was determined by enzymatic analysis (R-Biopharm, Milan, Italy).

2.3. Influence of pH on cholesterol solubility

The effect of low pH values, as those achieved by the acidifying bacterial activity, on the solubility of cholesterol was investigated. Three glass tubes containing 5 mL of MRS broth were supplemented with cholesterol to obtain a final concentration of 0.012% (w/v), acidified with 3.6% (w/w) HCl at pH 4 and incubated for 24 h at 37 °C. Then the cholesterol content was determined as previously described.

2.4. Influence of Tween 80/ethanol mixture on bacterial growth

As cholesterol was supplemented to MRS broth with a mixture of Tween 80/ethanol, the influence of these components on the growth of the strains was evaluated. The strains were inoculated at 2% in MRS broth, in MRS broth with Tween 80/ethanol mixture and in MRS broth with the cholesterol solution in Tween 80/ethanol mixture, and incubated at 37 °C for 24 h. Then, 1-mL samples were serially diluted in Ringer's solution (Oxoid, Milan, Italy) and plated in triplicate onto MRS agar (Oxoid, Milan, Italy). The plates were incubated at 37 °C for 24 h before enumeration and the tests were carried out in triplicate.

2.5. Cholesterol binding assay

The in vitro cholesterol binding assay was performed according to Hosono and Tono-Oka [13] modified protocol. Each strain was grown in 150 mL MRS broth at 37 °C for 24 h. Then, the cells were harvested by centrifugation at 1118× *g* for 10 min, washed twice with 0.9% (w/v) NaCl (Fluka, Milan, Italy) sterile solution, freeze-dried and kept at room temperature until use. A total of 330 mg of lyophilized cells was obtained for *L. plantarum* A110 and 300 mg for *L. plantarum* A106.

Ten milligrams of lyophilized cells were suspended in 1 mL of cholesterol-ethanol solution (100 μg of cholesterol dissolved in 1 mL of 60% ethanol), vortexed and incubated at 37 °C for 1 h in a shaking water bath. The mixture was then centrifuged at 1118× *g* for 10 min, and unbound cholesterol in the supernatant was determined by enzymatic analysis and the tests were carried out in triplicate.

2.6. Bile tolerance

Bile tolerance of strain was evaluated as rapidity of growth in a broth medium with and without bile acids as described by Gilliland and Walker [8]. Briefly, overnight cultures were inoculated at 1% in MRS broth and in MRS broth containing 0.3% (w/v) oxgall, incubated at 37 °C for 8 h and monitored hourly for growth spectrophotometrically at 620 nm. Comparison of cultures was based on their growth rates in each broth and the tests were repeated twice.

2.7. Acid tolerance

Assay was carried out as described by Pereira and Gibson [21]. Briefly, overnight

cultures of strains were inoculated at 10% into MRS broth that was previously adjusted to pH 2.0 with 3.6% (w/v) HCl (Fluka, Milan, Italy). The cultures were incubated anaerobically at 37 °C for 2 h. 1-mL samples were taken at various times (0, 30 and 120 min), serially 10-fold diluted in an anaerobic diluent (half-strength peptone water plus 0.5 g of L-cysteine $HCl{\cdot}L^{-1}$, pH 7.0) and plated in duplicate onto MRS agar (Oxoid, Milan, Italy). The plates were incubated at 37 °C for 24 h under anaerobic conditions before enumeration. The experiments were repeated twice.

2.8. Statistical analysis

Data (percentages of cholesterol reduction and log colony forming unit (CFU) values) were processed using ANOVA and the Duncan mean comparison test to highlight the differences in the cholesterol-lowering activity and the growth conditions among strains. Calculation was performed by Statistica 7.0 Software (Statsoft, Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

Table I shows the percentages of the cholesterol reduction in MRS broth for all the strains used in this study and the pH values of the cultures after 24 h of incubation. Two *L. plantarum* and three *L. paracasei* strains actively consumed cholesterol from the medium during static growth, in the absence of bile salts. Results from the test on the influence of pH on cholesterol solubility showed that after 24 h at 37 °C, the reduction of cholesterol concentration in MRS broth was not observed despite the acidification. Thus in the current experimental conditions, precipitation of cholesterol due to the acidic conditions did not occur, and its decrease could only be attributed to the bacterial growth. Moreover, different from the other examined bacterial species [3, 7, 8, 25], the six lactobacilli dairy cultures, including *L. acidophilus* LA-5®, which were able to decrease cholesterol (Tab. I), did not need bile salts as necessary components to the removal process.

Table I. Mean values of cholesterol reduction (%) for each examined strain in MRS broth after 24 h of incubation, standard deviation (n = 3), pH values and the result of variance analysis with the Duncan test performed on cholesterol reduction values obtained for each strain.

LAB	Cholesterol reduction (%)	pH
L. acidophilus LA-5®	14.5 ± 3.4b	3.99
L. plantarum A101	ND	3.89
L. plantarum A102	ND	3.93
L. plantarum A103	ND	3.91
L. plantarum A104	ND	4.01
L. plantarum A106	18.4 ± 0.9a	3.90
L. plantarum A110	20.5 ± 1.9a	3.84
L. plantarum A1010	ND	3.90
L. plantarum B102	ND	3.90
L. paracasei 24	8.6 ± 2.5c	3.84
L. paracasei 37	7.9 ± 1.9c	3.89
L. paracasei 38	6.5 ± 4.8c	3.86
L. paracasei 39	ND	3.93
L. paracasei 50	ND	3.84

Mean values with different letters differ for $P < 0.05$. ND, no cholesterol removal observed.

The two *L. plantarum* strains lowered the cholesterol content by an average of 19.4%, whereas the three *L. paracasei* strains lowered it by an average of 6.8%. However, the other six *L. plantarum* and two *L. paracasei* strains did not drop cholesterol at all. Generally, the cholesterol reduction of each culture among the three replicates has been very similar as indicated by the small associated standard deviations, except for the cholesterol decrease registered by *L. paracasei* 38 strain that was highly dependent on repetitions. The control strain *L. acidophilus* LA-5® showed an ability to decrease the cholesterol concentration in MRS broth, but the obtained values

Table II. Effect of Tween 80/ethanol mixture and cholesterol solution on *L. plantarum* strain growth and results of variance analysis performed on log values for each strain among the three culture media. Plate counts were expressed as $\log_{10}$ CFUs·mL^{-1}.

	MRS	MRS + T80E	MRS + CHT80E	Significance
L. plantarum A106	9.52 ± 0.40	8.92 ± 0.70	8.79 ± 0.60	ns
L. plantarum A110	9.02 ± 0.70	8.83 ± 0.50	8.72 ± 0.50	ns

T80E, Tween 80/ethanol mixture; CHT80E, cholesterol solution in Tween 80/ethanol and ns, not significant.

were lower than those shown by *L. plantarum* strains. These results showed that the cholesterol reduction was associated with growing cells and was strain specific; in this study, in fact a significant ($P < 0.05$) difference between the two studied species, *L. plantarum* and *L. paracasei*, was observed in the amount of the cholesterol removed. Within the same species, strains with and without cholesterol-lowering activity were found. However, statistical analysis did not show significant differences among the two strains of *L. plantarum* and among the three strains of *L. paracasei* that decreased cholesterol in MRS broth.

To elucidate which mechanism was involved in the cholesterol removal performed by the two active *L. plantarum* strains, a binding assay was carried out. The assay was not carried out on *L. acidophilus* as many works in the literature dealt with mechanisms involved in its lowering action. Data from the binding assay showed that cholesterol may have been merely adsorbed onto the cell surface; in fact both *L. plantarum* strains exhibited high binding ability with 75.6 ± 1.1% cholesterol bound to the cell preparation of *L. plantarum* A106 and 75.4 ± 1.6% to that of *L. plantarum* A110, without significant statistical differences among these two strains.

Comparison among *L. plantarum* A106 and *L. plantarum* A110 cell counts after 24 h of incubation in MRS broth, in MRS broth with Tween 80/ethanol mixture and in MRS broth with the cholesterol solution in Tween 80/ethanol showed that differences among CFU log values were not significant, and thus the presence of these components did not influence the growth of lactobacilli (Tab. II).

The same two *L. plantarum* strains that showed the highest cholesterol-lowering action in MRS broth were also tested in milk. To avoid milk coagulation that interfered with the cell separation from milk the cholesterol reduction was evaluated in milk after 8 h of incubation, but strains did not show removal activity despite the ability of these strains to decrease cholesterol in MRS broth after only 8 h of growth (data not shown). To obtain a cholesterol-lowering activity in milk, an incubation of 24 h was required. *L. acidophilus* LA-5® decreased the cholesterol content by an average of 9.4 ± 1.8%, *L. plantarum* A106 by an average of 8.2 ± 2.2% and *L. plantarum* A110 by an average of 5.0 ± 3.5%, without significant statistical differences among the three strains. Thus in MRS the efficacy of the strains isolated from cheese to remove cholesterol was higher than that of the reference, whereas in milk the cholesterol-lowering activity was approximately the same for both the *L. acidophilus* LA-5® and the two *L. plantarum* strains. The percentages of the cholesterol reduction in milk were less than those found in MRS broth probably due to the lower bioavailability of the cholesterol in milk, where it is partially incorporated into the fat globules.

Comparison between the values of cholesterol reduction found in this work with

those reported in the literature [1, 2, 4, 8, 18, 20] is not possible as different experimental conditions were used. In particular, published works are generally devoted to define the capability of different microorganisms to reduce cholesterol at the intestinal level using culture media enriched in bile salts and different cholesterol sources, incubation times, origin of strains and so on.

As these strains showed a cholesterol-lowering activity in both MRS and milk media, the survival capacity at low pH and the tolerance to bile salts were studied to evaluate their use as probiotic cultures or as non-starter LAB for the production of probiotic fermented milk [24]. The time required for both strains, *L. plantarum* A110 and *L. plantarum* A106, to increase the absorbance by 0.3 units was 3 h (data not shown) for both MRS and MRS with bile salts, showing the ability to easily grow in the presence of these components; in addition, the strains showed the same degree of survival at pH 2.0 for 3 h, demonstrating the capability to tolerate acidic conditions. In fact, *L. plantarum* A106 showed a viable cell count ($\log_{10}$ CFUs·mL^{-1}) of 8.34 ± 0.03 at 0 min, 8.34 ± 0.02 at 30 min and 8.33 ± 0.01 at 120 min and *L. plantarum* A110 showed a viable cell count of 8.33 ± 0.04 at 0 min, 8.34 ± 0.03 at 30 min and 8.37 ± 0.02 at 120 min.

In conclusion in this work, the dairy isolates belonging to the species *L. plantarum* and *L. paracasei* were examined to check their ability to lower cholesterol during growth in MRS broth and milk. Our results demonstrated that the cholesterol removal can also take place in the absence of bile salts. This capability was exploited during fermentation of milk, where the cholesterol-lowering activity was maintained. Thus on the one hand, since these *L. plantarum* strains also grew with bile salts and low pH, they could be considered as probiotics establishing the effective cholesterol absorption property after acid and bile salts exposition. On the other hand, to develop a new functional food with low cholesterol content, these results must be complemented with technological assays.

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