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Note

Fermented milks from *Enterococcus faecalis* TH563 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LA2 manifest different degrees of ACE-inhibitory and immunomodulatory activities

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Abstract – Milk proteins are precursors of biologically active components that are released by enzymatic proteolysis. Among the biological activities recognised in milk components, the angiotensin-I converting enzyme (ACE)-inhibitory and immunomodulatory activities are of great interest. In the present work the ACE-inhibitory and immunomodulatory activities were analysed in milks fermented by two bacterial strains isolated from Italian dairy products, *Enterococcus faecalis* TH563 or *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. delb. bulgaricus*) LA2. ACE-inhibitory activity was determined by an already established enzymatic method and immunomodulatory activity by the proliferation of bovine peripheral blood lymphocytes (BPBL) taken from nine cows. BPBL were incubated for 48 h with increasing concentrations of peptide fractions (< 5000 g·mol^{-1}) extracted from the two fermented milks. Concanavalin A (conA), a known activator of lymphocyte proliferation, was used as a positive control. Fermentation products from *E. faecalis* TH563 showed a significantly ($P < 0.05$) greater ACE-inhibitory activity than that obtained by *L. delb. bulgaricus* LA2 (69.43 ± 3.12% vs. 60.86 ± 1.01%). The immunomodulatory activity showed a large interanimal variability. Peptide fractions from milk fermented by *L. delb. bulgaricus* LA2 significantly inhibited BPBL proliferation at concentrations of 5, 25 and 50 µg·mL^{-1} in the presence of conA ($P < 0.01$). *E. faecalis* TH563 did not significantly modify BPBL proliferation at any peptide concentration used. In conclusion, *L. delb. bulgaricus* LA2-fermented milk showed ACE-inhibitory and immunomodulatory activities, while *E. faecalis* TH563-fermented milk had high ACE-inhibitory activity, suggesting a possible use of these strains for determining bioactive properties in dairy products.

fermented milk / *Enterococcus faecalis* / *Lactobacillus delbrueckii* subsp. *bulgaricus* / ACE-inhibitory activity / immunomodulatory activity

摘要 – *Enterococcus faecalis* TH563 和 *Lactobacillus delbrueckii* subsp. *bulgaricus* LA2 发酵乳的 ACE 抑制活性和免疫调节活性。乳蛋白水解后的化合物是生物活性成分的前体，在这些生物活性化合物中，具有血管紧缩素-I 转移酶 (ACE) 抑制活性和免疫调节活性的化合物

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引起人们广泛的关注。以分离于意大利乳制品的两株乳酸菌 *Enterococcus faecalis* TH563 和 *Lactobacillus delbrueckii* subsp. *bulgaricus* LA2 为目标菌株，研究了两株菌发酵乳制品的 ACE 抑制活性和免疫调节活性。采用酶法测定 ACE 抑制活性，以及根据牛外周血淋巴细胞 (BPBL) 增殖实验来评价免疫调节活性。细胞分别在有和无淋巴细胞增殖激活剂伴刀豆球蛋白 A (conA) 的两种发酵奶中孵化 48 h。*E. faecalis* TH563 发酵乳比 *L. delb. bulgaricus* LA2 发酵乳的 ACE 抑制活性 (69.43 ± 3.12%; $P < 0.05$) 高。但是免疫调节活性在菌种之间表现出较大的可变性，含 conA 的 *L. delb. bulgaricus* LA2 发酵乳 (5–25–50 μg·mL^{-1}) 可以显著地抑制 BPBL 的增殖 ($P < 0.01$)，而在任何浓度下的 *E. faecalis* TH563 发酵乳都不能影响细胞的增殖。因此，*L. delb. bulgaricus* LA2 发酵乳具有 ACE 抑制活性和免疫调节活性，而 *E. faecalis* TH563 发酵乳只具有 ACE 抑制活性。因此这两株乳酸菌具有潜在用于乳品工业中生产生物活性肽。

发酵乳 / *Enterococcus faecalis* / *Lactobacillus delbrueckii* subsp. *bulgaricus* / ACE 抑制活性 / 免疫调节活性

Résumé – Les laits fermentés par *Enterococcus faecalis* TH563 et *Lactobacillus delbrueckii bulgaricus* LA2 montrent différents degrés d'activités anti-ACE et immunomodulatrice. Les protéines du lait sont des précurseurs de composés à activité biologique, qui sont libérés par protéolyse enzymatique. L'inhibition de l'enzyme convertissant l'angiotensine-I (ACE) et l'activité immunomodulatrice sont des activités d'intérêt parmi les activités biologiques reconnues des composés du lait. Dans cette étude, les activités anti-ACE et immunomodulatrices ont été analysées dans du lait fermenté par deux souches bactériennes isolées de produits laitiers italiens, *Enterococcus faecalis* TH563 ou *Lactobacillus delbrueckii* ssp. *bulgaricus* LA2. L'activité anti-ACE était déterminée par une méthode enzymatique pré-établie, l'activité immunomodulatrice par la prolifération de lymphocytes de sang périphérique de bovin (BPBL), prélevés à partir de neuf vaches. Les BPBL étaient incubées pendant 48 h en présence de concentrations croissantes de fractions peptidiques (< 5000 g·mol^{-1}) extraites des deux laits fermentés. La concanavalin A (conA), un activateur connu de la prolifération des lymphocytes, était utilisée comme témoin positif. Le produit fermenté par *E. faecalis* TH563 montrait une activité anti-ACE significativement ($P < 0,05$) plus élevée que celle obtenue avec *L. delb. bulgaricus* LA2 (69,43 ± 3,12 %, vs. 60,86 ± 1,01 %). L'activité immunomodulatrice montrait une forte variabilité inter-animal. Les fractions peptidiques issues du lait fermenté par *L. delb. bulgaricus* LA2 inhibaient significativement ($P < 0,01$) la prolifération des BPBL aux concentrations 5, 25, et 50 μg·mL^{-1} en présence de conA. *E. faecalis* TH563 ne modifiait pas significativement la prolifération des BPBL quelle que soit la concentration en peptides mise en œuvre. En conclusion, *L. delb. bulgaricus* LA2 produisait un lait fermenté avec des activités anti-ACE et immunomodulatrices, alors que *E. faecalis* TH563 produisait un lait fermenté à forte activité anti-ACE, suggérant une utilisation possible de ces souches pour apporter des propriétés bioactives dans les produits laitiers.

lait fermenté / *Enterococcus faecalis* / *Lactobacillus delbrueckii* subsp. *bulgaricus* / activité anti-ACE / activité immunomodulatrice

1. INTRODUCTION

There is evidence that several foods or foods ingredients provide a benefit beyond the nutrients they contain. These substances are defined as functional food, and their putative biological effects have been extensively studied. To date, antihypertensive and immunomodulatory bioactivities are frequently exploited in the production of foodstuffs formulated to provide putative health benefits [11, 18].

Interestingly, angiotensin-I converting enzyme (ACE)-inhibitory and immunomodulatory properties seem to be associated, possibly because both are correlated to the presence of short-chain peptides [22].

So far, lactic acid bacteria have been preferred to other microorganisms to produce fermented milks rich in ACE-inhibitory activity [6, 23], in particular *Lactobacillus helveticus* [16, 20, 21], *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. delb. bulgaricus*) and *Lactococcus lactis* subsp. *cremoris* (*L. lactis cremoris*) [10]. Moreover, some bacterial strains, mostly lactic acid bacteria, release components during fermentation that possess immunomodulatory activity [24, 26]. Lactic acid bacteria fermentation products potentiate the cell-mediated immune response by increasing the proliferative response of lymphocytes to concanavalin A (conA), a known activator of lymphocyte proliferation [5]. In addition, some findings suggest that milk fermented by *Lactobacillus* strains can modulate the immune response against breast cancer cells in mice [26] and improve innate-defense capacity in human being [24].

However, species other than those belonging to *Lactobacillus* genus are often isolated from dairy products, that may possess interesting properties [6, 12, 23]. We were interested in *Enterococcus faecalis* because it is an enterococcal species frequently found in dairy products, traditional cheeses in particular, where it may play an important role in determining cheese taste and texture [1, 27].

The aim of our study was to measure the ACE-inhibitory and immunomodulatory bioactivities in milk fermented with *E. faecalis* TH563 and compare them to those generated by *L. delb. bulgaricus* LA2. These strains belong to a panel of 14 bacterial strains (7 *L. delb. lactis*, 2 *L. delb. bulgaricus*, 1 *L. helveticus*, 2 *L. paracasei* and 2 *E. faecalis*) representing species that are frequently isolated from traditional dairy products of North Eastern Italy [2] and showing different degrees of proteolytic activity.

Although *E. faecalis* is reported to generate fermented milk with ACE-inhibitory activity [17, 19, 25], few information about its ability to generate immunomodulatory activity is available. On the contrary, *L. delb. bulgaricus* is commonly used as starter culture for the production of yogurt and fermented milks.

2. MATERIALS AND METHODS

2.1. Bacteria culture

E. faecalis TH563 and *L. delb. bulgaricus* LA2 were evaluated for their proteolytic activity as described by Hull [13] and in accordance with IDF, Standard 149A [14].

Lactobacilli were propagated in MRS (de Man, Rogosa and Sharpe) broth (Biolife, Milan, Italy) for 24 h at 44 °C, while enterococci were propagated in M17 broth (Difco Laboratories, Detroit, Michigan) for 24 h at 37 °C. Revitalised microorganisms were used to inoculate (1%, v/v) 10 mL of sterilised skim milk (Biolife, Milan, Italy), which was incubated for 24 h at 44 °C (lactobacilli) and 37 °C (enterococci). One millilitre of these milk pre-cultures was used to inoculate 100 mL of skim milk. Incubation was carried out under sterile conditions at 44 °C (lactobacilli) and 37 °C (enterococci).

2.2. Separation of the peptide fraction

Fermented milk samples were centrifuged at 20 000× *g* for 15 min at 15 °C (J2-21 Beckman Coulter centrifuge, JA 20 rotor, Fullerton, CA, USA) to remove bacterial debris. The supernatant was filtered with Amicon Centricon Ultra15 (molecular weight cut-off 5000 g·mol^{-1}; Millipore, Billerica, MA, USA) by centrifugation at 3200× *g* for 40 min at 15 °C. The fraction with molecular weight lower than 5000 g·mol^{-1} (5000 g·mol^{-1} fraction) was stored at −20 °C and used for further analyses. The concentration of peptides in the 5000 g·mol^{-1}

fractions was spectrophotometrically determined by the method of Layne [15].

2.3. ACE-inhibitory activity

The ACE-inhibitory activity of the 5000 g·mol^{-1} fractions was measured by the method of Cushman and Cheung [4], as modified by Nakamura et al. [20]. An Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech, NJ, USA) was used to measure the optical density of each 5000 g·mol^{-1} fraction.

Each test was performed in triplicate, and the measured absorbance was used for the calculation of the percentage of ACE inhibition (% ACE-I) as follows:

$$\%\ \text{ACE-I} = 100 \times (B - A)/(B - C),$$

where A is the optical density of the samples in the presence of ACE, B is the optical density of the total activity and C is the optical density of the blank. Data were subjected to the analysis of variance, and the differences between mean values were analysed by the test of Duncan (SPSS Inc., Chicago, IL, USA).

2.4. Bovine peripheral blood lymphocytes proliferation

Ten millilitres of 5000 g·mol^{-1} fraction of fermented milk by *E. faecalis* TH563 and 30 mL of 5000 g·mol^{-1} fraction of fermented milk by *L. delb. bulgaricus* LA2 were dried under vacuum, and the obtained powders were dissolved in 5 mL of complete medium prepared as follows: RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% of heat-inactivated new-born calf serum (NCS, Sigma, St. Louis, MO, USA), 2 mmol·L^{-1} of L-glutamine (Sigma, St. Louis, MO, USA), 100 μg·mL^{-1} of streptomycin and 100 U·mL^{-1} of penicillin (Sigma, St. Louis, MO, USA). The concentration of peptides in the 5000 g·mol^{-1} fraction for the proliferation test was determined spectrophotometrically as described by Layne [15]. The 5000 g·mol^{-1} fractions were sterilised by filtration (0.22 μm filters) and stored at −20 °C until use.

To evaluate the immunomodulatory activity of the 5000 g·mol^{-1} fractions, bovine peripheral blood lymphocytes (BPBL) were isolated from whole heparin-anticoagulated blood of nine non-pregnant, non-lactating dairy cows without clinical symptoms by density gradient centrifugation using the Lymphoprep reagent (AXIS-SHIELD PoC AS, Oslo, Norway). Cells were suspended in complete medium in the presence of 2 μg·mL^{-1} of conA (Sigma, St. Louis, MO, USA) as mitogen and were incubated at 37 °C in 5% CO_2. After 24 h of differentiation, non-adherent BPBL were separated from adherent leukocytes and tested for viability with Trypan blue staining. Viable BPBL were adjusted at a density of 3×10^6 cells·mL^{-1} in complete medium and incubated for 48 h in a 96-well microplate (100 μL cell suspension per well) with or without conA (2 μg·mL^{-1}, positive control) and in the presence of increasing concentrations (from 0 μg·mL^{-1} to 100 μg·mL^{-1}) of each fermented milk. At the end of the incubation period, proliferation test was assessed by 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation test, following the manufacturer's instructions. Briefly, MTT powder (Sigma, St. Louis, MO, USA) was dissolved in Hanks' Balanced Salt Solution (Gibco Invitrogen, UK) (5 mg·mL^{-1}), added to the cells (15 μL per well) and incubated for 3 h to allow the reductases of living cells to convert the MTT into the insoluble formazan. The formazan was then eluted with 10% (v/v) Triton X100 (Sigma, St. Louis, MO, USA), and the absorbance was measured at a wavelength of 570 nm with background subtraction at 630 nm using a microplate reader (Spectra Count, Packard Bioscience).

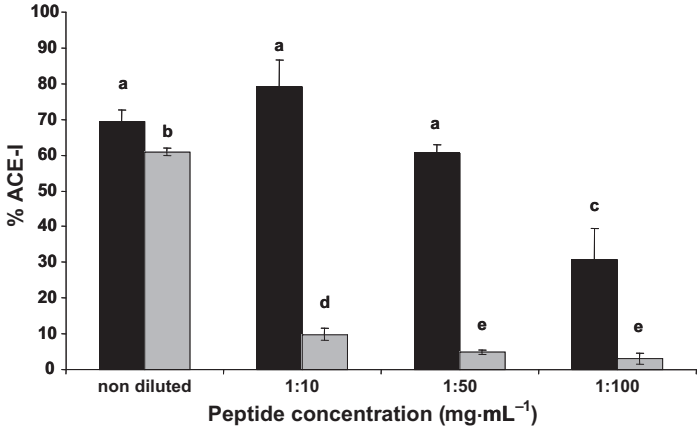

Figure 1. ACE-inhibitory activity of the 5000 g·mol^{-1} fraction obtained after Amicon Ultra15 filtration of fermented milks. ACE-inhibitory activity was expressed as the % ACE-I. Milk fermented by *E. faecalis* TH563 (dark grey bars) showed a higher ACE-inhibitory activity if compared to *L. delb. bulgaricus* LA2 (light grey bars). Results are presented as means ± SEM of three independent experiments. Different superscripts indicate statistically different means ($P < 0.05$; Duncan test).

Each cell proliferation test was performed in triplicate. The results were expressed as the percentage of the optical density observed in the conA-treated BPBL (% conA). Relative variations of cellular proliferation produced by each fermented milk were analysed using a generalised linear model (GLM, SPSS Inc., Chicago, IL, USA). Differences between mean values were analysed by the Dunnett test (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

Milk fermented by *E. faecalis* TH563 showed a significantly ($P < 0.05$) higher ACE-inhibitory activity (69.43 ± 3.12%) than *L. delb. bulgaricus* LA2 (60.86 ± 1.01%). The persistency of high ACE-inhibitory values up to 1:50 dilution for *E. faecalis* TH563 indicated an enzyme saturation effect that disappeared at 1:100 dilution. On the contrary, ACE-inhibitory activity in milk fermented by *L. delb. bulgaricus* LA2 was significantly reduced to very low levels when the 5000 g·mol^{-1} fraction was diluted 10-fold ($P < 0.05$) (Fig. 1).

Even if strains of *E. faecalis* have been reported to possess high proteolytic activity [27], the ability to produce fermented milks with ACE-inhibitory activity has been scarcely documented [19, 25]. In the present experiment, ACE-inhibitory activity seemed to be positively related to the proteolytic activity of the strain of interest. In fact, *E. faecalis* TH563 showed a higher proteolytic activity (0.292 mg of tyrosine·mL^{-1}) and peptide concentration (14.78 mg·mL^{-1}) in the 5000 g·mol^{-1} fraction than *L. delb. bulgaricus* LA2 (proteolytic activity: 0.100 mg of tyrosine·mL^{-1}, peptide concentration: 4.89 mg·mL^{-1}), suggesting potentially greater ability to produce small peptides, which are mainly responsible for ACE-inhibitory activity [29].

The peptide concentration in the samples for MTT was 30.43 mg·mL^{-1}and 37.72 mg·mL^{-1} for *E. faecalis* TH563 and *L. delb. bulgaricus* LA2, respectively.

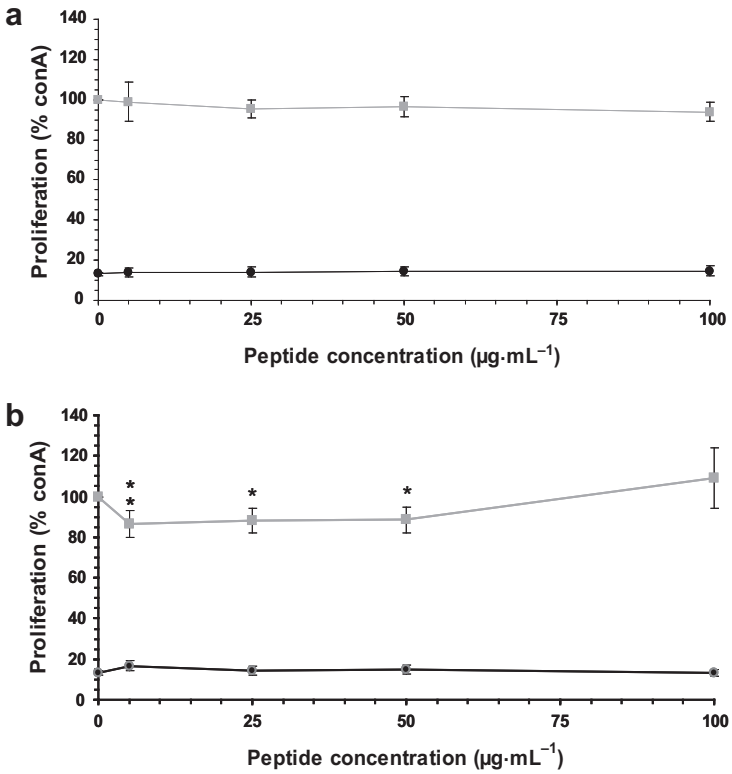

Figure 2. Dose-response effect of 5000 g·mol^{-1} fraction obtained after Amicon Ultra15 filtration from milk fermented by *E. faecalis* TH563 (a) or *L. delb. bulgaricus* LA2 (b) by MTT proliferation test, in the presence (■) or in the absence (●) of the mitogen concanavalin A (conA). The data were expressed as the percentage of the optical density observed in conA-treated BPBL cultured without fermented milk but in the presence of conA (positive control). Results are presented as means ± SEM of nine independent experiments for each strain. Asterisks indicate means significantly different from the positive control (*$P < 0.001$; **$P < 0.01$; Dunnett *t*-test).

The 5000 g·mol^{-1} fraction obtained from the milk fermented by *E. faecalis* TH563 did not significantly affect BPBL proliferation either with or without the mitogen conA (Fig. 2a). The 5000 g·mol^{-1} fraction obtained from the milk fermented by *L. delb. bulgaricus* LA2 was able to decrease the conA-induced BPBL proliferation when added at 5 µg·mL^{-1} ($P < 0.001$), 25 µg·mL^{-1} and 50 µg·mL^{-1} ($P < 0.01$) peptide concentration, but not at 100 µg·mL^{-1} (Fig. 2b). At this concentration, other factors might be present in a sufficient concentration to counteract the inhibitory effect on BPBL proliferation. Moreover, it is difficult to explain how fermented milks could modulate the cells of the immune system, and it is even more complicated to identify specific components produced during milk fermentation responsible for these immunomodulatory activities. Fermented milks are complex matrices, rich not only in proteins and peptides but also in sugars, fat, minerals and

polysaccharides of the bacterial membrane that can contribute to the whole immunomodulatory effect. In this regard, it was demonstrated that milk fatty acids produced during fermentation affect cellular proliferation [7].

When the milk fermented by *L. delb. bulgaricus* LA2 was administered without conA, it did not affect BPBL proliferation, although a slight increase in BPBL proliferation was observed at a peptide concentration of 5 $\mu g \cdot mL^{-1}$ (Fig. 2b).

The results of this experiment were in agreement with the hypothesis of Fujiwara et al. [9] suggesting that immunomodulatory activity is essentially expressed by strains of lactobacilli. Conversely, the immunomodulatory activity was not associated with ACE-inhibitory activity, differently from the assumption of Narva et al. [22].

The preliminary results of our work suggest that the presence of *E. faecalis* strains in traditional cheeses, where they play an important role in determining cheese taste and texture [1, 27], could contribute to generate dairy products with ACE-inhibitory activity. *E. faecalis* strains are not usually employed in the production of dairy foods since some of them can harbour potential virulence factors or antibiotic resistance [28], and their presence in the food system is still a matter of controversy due to their pathogenic potential [8]. Thus, *E. faecalis* strains should be evaluated for safety aspects before being used in the food industry. *E. faecalis* TH563 does not carry *van*A or *van*B genetic determinants for vancomycin transferable antibiotic resistance [2], but in order to completely assess its safety as adjunct culture in fermented milk, the strain should be tested for the absence of other potential virulence factors such as haemolysin, aggregation substances, surface proteins *ace* and *esp* [3].

Finally, it would be interesting to evaluate if milk fermented with both *E. faecalis* TH563 and *L. delb. bulgaricus* LA2 as mixed culture could generate a fermented milk showing both ACE-inhibitory and immunomodulatory activities.

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