

The role of ferulic acid esterase in the growth of *Lactobacillus helveticus* in the presence of phenolic acids and their derivatives

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Abstract The goal of this study was to determine the ability of benzoic and cinnamic acid derivatives ($n = 38$) as inhibitors of the growth of the T103 and T159 strains of *Lactobacillus helveticus*. The compounds studied inhibited the growth of *Lb. helveticus* T159 more effectively, whereas as many as 22 other compounds tested had no effect on the growth of *Lb. helveticus* T103. In numerous cases, the minimal inhibitory concentration values of cinnamic acid derivatives were lower than those of their corresponding benzoic counterparts. Ethyl ferulate, ethyl vanillate and ethyl 4-hydroxybenzoate exhibited significantly higher ($p < 0.05$) growth-inhibitory activity towards *Lb. helveticus* T159 than corresponding free phenolic acids. The ability of *Lb. helveticus* T159 to synthesize ferulic acid esterase and surface proteins (S-layer) was reported, whereas no such ability was observed in the case of the other bacterial strain. Therefore, it can be speculated that the differences in the inhibition of both strains can be, at least partly, attributed to the presence of the enzyme and/or hydrophobic surface proteins. The molecular composition of free phenolic acids and their derivatives (the number and/or position of OH or CH₃) seemed to play a minor role in this regard.

Keywords *Lactobacillus helveticus* · Phenolic acids · Antibacterial effect · Ferulic acid esterase · S-layer protein

Introduction

Lactobacillus helveticus strains are of considerable technological importance because of their role in the manufacture of many food products. They also play an important role as probiotic gastrointestinal microflora [1]. It was observed that a significant amount of the phenolic acids in the human diet is not directly absorbed and thus can modify the growth of gut microbiota [2]. Few studies have been conducted to investigate the influence of phenolic acids on intestinal bacteria. The growth-inhibitory activity of phenolic acids originating from plant and dairy sources on lactic acid bacteria from the genus *Lactobacillus* has been noted in detailed reviews by Rodríguez et al. [3] and Requena et al. [4]. However, previous papers have studied a limited number of phenolic acids. It should also be noted that selected *Lactobacillus* strains are able to release free phenolic acids from plant material via the action of ferulic acid esterase (FAE); and as a consequence, the concentration of active, free forms of phenolic acids in the environment is increased. We have previously purified and characterized FAE from *Lb. acidophilus* K1 [5]. The biotransformation of phenolic acids by *Lb. acidophilus* K1 was also observed in previous studies [6]. It has been reported that phenolic compounds may be detoxified by forming complexes with negatively charged carboxyl and phosphorous residues of the S-layer protein [7]. S-layers have been found in a few strains of *Lb. helveticus* [1, 8]. However, our results have implicated that not all *Lb. helveticus* strains are able to synthesize S-layer proteins and FAE (data not shown). The work hypothesis of this study was to investigate a potential link between the susceptibility of the strain to phenolic acids in relation to the presence/absence of FAE activity and S-layer. One approach to solve this problem is the construction of S-layer and FAE isogenic mutants. Unfortunately, in

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lactobacilli, the construction of stable S-layer knock-out mutants has so far not been achieved [9]. The second approach (which was exploited in this work) is the use of a selected strain producing S-layer and FAE and the reference strain with no ability to synthesize S-layer proteins and the enzyme.

The present study was undertaken to investigate the effect of single phenolic acids or their derivatives on the growth of *Lb. helveticus* strains. An attempt was made to explain the significance of simultaneous presence of FAE and S-layer proteins on the growth of bacteria.

Materials and methods

Reagents

The following test compounds were purchased from Sigma-Aldrich (Poznań, Poland): benzoic acid (242381), 2-hydroxybenzoic acid (84210), 3-hydroxybenzoic acid (H20008), 4-hydroxybenzoic acid (H20059), 4-aminobenzoic acid (429767), ethyl 4-hydroxybenzoate (75769), 4-hydroxyphenylacetic acid (H50004), 4-hydroxyphenylpyruvic acid (I14286), 2,3-dihydroxybenzoic acid (126209), 2,4-dihydroxybenzoic acid (D109401), 2,5-dihydroxybenzoic acid (149357), 2,5-dihydroxyphenylacetic acid (H0751), 3,4-dihydroxybenzoic acid (37580), 3,4-dimethoxybenzoic acid (D131806), 3,5-dihydroxybenzoic acid (D110000), 3,4,5-trihydroxybenzoic acid (G7384), 4-hydroxy-3-methoxybenzoic acid (94770), 4-hydroxy-3-methoxyphenylacetic acid (H1252), ethyl 4-hydroxy-3-methoxybenzoate (S459267), 4-hydroxy-3,5-dimethoxybenzoic acid (S6881), cinnamic acid (C80857), 2-hydroxycinnamic acid (H22809), 3-hydroxycinnamic acid (H23007), 4-hydroxycinnamic acid (C9008), 3,4-dihydroxycinnamic acid (C0625), 3,4-dimethoxycinnamic acid (D133809), 3,4-dihydroxyhydrocinnamic acid (102601), 3-caffeoylquinic acid (C3878), 2,3-dicaffeoyl-L-tartaric acid (C7243), 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (536954), 4-hydroxy-3-methoxycinnamic acid (128708), 3-hydroxy-4-methoxycinnamic acid (103012), ethyl 4-hydroxy-3-methoxycinnamate (320617), 4-hydroxy-3,5-dimethoxycinnamic acid (D7927), carnosic acid (C0609), nordihydroguaiaretic acid (74540) and ellagic acid (E2250). Methyl esters of phenolic acids were purchased from Apin Chemicals Ltd (Abingdon, UK): methyl 4-hydroxycinnamate (16975 m), methyl 4-hydroxy-3-methoxycinnamate (02838 m), methyl 4-hydroxy-3,5-dimethoxybenzoate (02865 m), methyl 4-hydroxy-3-methoxybenzoate (02868 m). HPLC grade chromatography reagents, as well as other reagents of analytical grade, were sourced from POCh Gliwice, Poland.

Bacterial strains and culture conditions

Eleven strains of *Lb. helveticus*, kindly provided by Prof. Łucja Łaniewska-Trokenheim (University of Warmia and Mazury in Olsztyn), were examined for the presence of S-layer proteins and FAE activity (data will be published soon). *Lb. helveticus* T159 was selected due to the ability to produce S-layer proteins and the highest FAE activity. The reference strain *Lb. helveticus* T103 used in this study had no ability to synthesize S-layer proteins and FAE. The strains had been isolated from Polish-fermented milk products and deposited in the Polish Microorganism Collection (Wrocław, Poland). Bacteria were stored and cultured in de Man, Rogosa and Sharpe (MRS) (BTL, Poland) broth supplemented with 0.5 g/L L-cysteine and were incubated in anaerobic conditions at 42 °C for 48 h. Anaerobic conditions were obtained by injecting 0.5 mL of 15 % (m/v) NaHCO₃ and 0.5 mL of 20 % (m/v) pyrogallol solutions in water into cotton stoppers followed by aseptic sealing of the tubes with parafilm.

Esterase activity assay

Strains were grown, as described Szwajgier and Jakubczyk [6], in the presence of either methyl ferulate or methyl vanillate (0.1 % m/v) as a carbon source. After 24 h, samples were centrifuged (10,000×g, 15 min, 4 °C). Cultivations were duplicated, and each cultivation was individually analysed for FAE activity. For this purpose, supernatants (0.5 mL) were mixed with 0.1 mL of methyl ferulate or methyl vanillate solution. Samples were incubated for 30 min at 42 °C followed by heating in boiling water (5 min), direct cooling on ice and filtration (0.45-μm syringe filters). Both blank samples lacking the substrate or study samples were run and analysed simultaneously, as described above. The released ferulic acid or vanillic acid was analysed with an HPLC–UV system consisting of: two Smartline 100 pumps, a dynamic mixer and a 20 μL loop (Knauer, Germany). Detection at 320 nm was performed using a UV–VIS detector (Linear California, USA) coupled with an IF2 interface (Knauer, Germany). For separations, a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 μm) with a Waters Symmetry precolumn was used. The gradient (1 mL/min) was formed by deionized water with 1 % (v/v) formic acid (A) and 90 % HPLC grade methanol in deionized water (B): 0–5 min 0 % B, 5–35 min 0–40 % B, 35–50 min 40–60 % B, 50–55 min 60–0 % B, 55–60 min 0 % B. Signals were analysed using the Eurochrom 3.05 P5 program. FAE activity was expressed in units. One unit was equal to 1 mM of ferulic acid released in 1 mL of the reaction medium after 1 min of incubation. The presented results are mean values with standard deviations obtained

after analysis performed on two separate cultivations of bacterial strains with the substrate.

Antimicrobial activity assay

Phenolic acids and their derivatives were tested using the microdilution broth method [10]. All compounds were dissolved in DMSO (Sigma, Germany), and dilutions of each stock solution (0.03–2000 $\mu\text{g}/\text{mL}$) were prepared in MRS broth supplemented with 0.5 g/L L-cysteine. The growth rate of each tested bacteria was monitored by measuring optical density (OD_{600}) using Bioscreen C (LabSystem, Finland) [11].

Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound that visibly inhibited the bacterial growth in comparison with the control. The MBC value was defined as the lowest concentration of the compound at which there was no bacterial growth. The experiment was performed in triplicate.

Detection and isolation of S-layer proteins

Bacterial cultures were adjusted to the optical density OD_{600} of 0.5 using phosphate-buffered saline, pH 7.2, and 1.0 mL of each culture was centrifuged (15,000 $\times g$, 4 $^{\circ}\text{C}$). The cell pellets were washed twice with deionised water and re-suspended in 30 μl SDS–PAGE sample buffer. The suspensions were heated for 5 min at 100 $^{\circ}\text{C}$ and centrifuged as above. Ten microlitres of the supernatants (which was equal to 25 μg of protein) were checked for the presence of S-layer proteins by SDS–PAGE, as described by Laemmli [12]. The gels were photographed using the Gel-Doc documentation system (Biorad, USA).

For isolation of S-layer proteins, a bacterial biomass from 500-mL cultures grown in MRS medium for 2 days at 42 $^{\circ}\text{C}$ in anaerobic conditions was harvested by centrifugation (9,000 $\times g$, 4 $^{\circ}\text{C}$) and washed twice with sterile deionised water. One gram of wet cells was extracted with 5.0 mL 5 M LiCl [13] for 2 h on a shaker (200 rpm), then centrifuged (30,000 $\times g$ for 15 min) and dialysed against deionised water at 4 $^{\circ}\text{C}$ for 48 h. Finally, the isolated cell envelope proteins were frozen at -80°C for 1 h and then freeze-dried in a freeze-dryer (Labconco, Kansas City, USA) at -50°C and 0.024 mBar for 18 h. The insoluble S-layer proteins were stored at -20°C .

Cell surface hydrophobicity assay

The hydrophobicity of the cell surface was determined using the salt aggregation test (SAT), as previously described [14]. To analyse the presence of specific hydrophobic cell surface proteins, a quantitative congo red binding assay (CRB) was performed, as described by Qadri et al. [15].

Statistical analysis

Mean values with standard deviations were presented. Tukey's HSD test (Statistica ver. 8, Cracow, Poland) was used for the analysis of results. Results presented in Table 1 were discussed on the basis of a significance level set at $p < 0.05$.

Results

FAE activity assay

FAE activity was detected only in *Lb. helveticus* T159. The enzyme activity was 0.16 ± 0.00 (0 h)– 19.57 ± 0.31 units (24 h) in the presence of methyl ferulate as a carbon source (Fig. 1). In *Lb. helveticus* T103, FAE activity was not detected in the presence of methyl ferulate. Similarly, no FAE activity was detected in supernatants obtained after the cultivations of both strains in the presence of methyl vanillate (Fig. 2).

The antibacterial activity of phenolic acids and their non-ester derivatives

The survival parameters MIC and MBC were used for the determination of the extent to which phenolic acids and their derivatives can affect the growth of *Lb. helveticus* strains (Table 1). After a short period of incubation, *Lb. helveticus* T159 was more sensitive to most of the compounds that belong to the group of benzoic as well as cinnamic acid derivatives, while 22 other compounds tested from both groups had no effect on the growth of *Lb. helveticus* T103. Interesting observations were made during the comparison of MIC and MBC values of selected free phenolic acids and their corresponding esters. A distinct difference was obtained when comparing the influence of vanillic acid (17) and its ethyl ester (20) on the growth of both strains. The MIC value of ethyl vanillate in *Lb. helveticus* T159 cultivations (0.03 $\mu\text{g}/\text{mL}$) was significantly lower than in the case of free vanillic acid, whereas the ester had no effect on the growth of *Lb. helveticus* T103. After 6 h of incubation with ethyl vanillate, a further inhibition of *Lb. helveticus* T159 growth was observed, but no reduction in T103 strain was seen. The MIC value for ethyl 4-hydroxybenzoate (5) was lower than the MIC of free phenolic acid (4) in case of *Lb. helveticus* T159 cultivated both for 2 h and 6 h. Moreover, the MBC of this ester was 2 $\mu\text{g}/\text{mL}$, whereas in the case of free 4-hydroxybenzoic acid MBC was not detected. In the case of two benzoic acid derivatives, 4-OH-phenylacetic acid (7) and methyl syringate (22), no inhibitory effect was observed towards both strains.

In general, in the case of both bacterial strains, the MIC and MBC values recorded for cinnamic acid derivatives

Table 1 Effect of phenolic acids and their derivatives on *Lb. helveticus* T159 and *Lb. helveticus* T103 growth

Compound	T159			T103		
	MIC ($\mu\text{g/mL}$)		MBC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)		(MBC $\mu\text{g/MI}$)
	2 h	6 h		2 h	6 h	
Benzoic acid and its derivatives						
1. Benzoic acid	1.00 \pm 0.03	0.50 \pm 0.03	2.00	2.00 \pm 0.06	0.50 \pm 0.02	–
2. 2-OH-benzoic acid (salicylic acid)	0.13 \pm 0.06	0.50 \pm 0.01	2.00	2.00 \pm 0.02	0.13 \pm 0.04	2.00
3. 3-OH-benzoic acid	1.00 \pm 0.07	0.50 \pm 0.02	2.00	2.00 \pm 0.05	0.50 \pm 0.02	–
4. 4-OH-benzoic acid	1.00 \pm 0.02	1.00 \pm 0.04	–	2.00 \pm 0.01	0.25 \pm 0.01	–
5. Ethyl 4-OH-benzoate	0.13 \pm 0.04	0.13 \pm 0.03	2.00	nd	0.25 \pm 0.05	–
6. 4-NH ₂ -benzoic acid (vitamin H ₁)	1.00 \pm 0.03	1.00 \pm 0.04	–	1.00 \pm 0.06	1.00 \pm 0.03	–
7. 4-OH-phenylacetic acid	nd	nd	–	nd	nd	–
8. 4-OH-phenylpyruvic acid	1.00 \pm 0.02	1.00 \pm 0.01	–	0.50 \pm 0.02	0.50 \pm 0.02	–
9. 2,3-di-OH-benzoic acid	0.13 \pm 0.03	0.13 \pm 0.03	–	1.00 \pm 0.09	0.13 \pm 0.01	2.00
10. 2,4-di-OH-benzoic acid (β -resorcylic acid)	0.25 \pm 0.02	0.25 \pm 0.01	2.00	0.13 \pm 0.05	0.13 \pm 0.05	–
11. 2,5-di-OH-benzoic acid (gentisic acid)	0.50 \pm 0.04	0.50 \pm 0.01	2.00	2.00 \pm 0.05	0.25 \pm 0.02	–
12. 2,5-di-OH-phenylacetic acid (homogentisic acid)	1.00 \pm 0.02	1.00 \pm 0.05	–	nd	1.00 \pm 0.06	–
13. 3,4-di-OH-benzoic acid (protocatechuic acid)	1.00 \pm 0.01	1.00 \pm 0.02	–	nd	2.00 \pm 0.01	–
14. 3,4-di-OCH ₃ -benzoic acid (veratric acid)	1.00 \pm 0.04	1.00 \pm 0.01	–	nd	0.50 \pm 0.01	–
15. 3,5-di-OH-benzoic acid (α -resorcylic acid)	0.13 \pm 0.00	0.13 \pm 0.02	–	nd	0.50 \pm 0.07	–
16. 3,4,5-tri-OH-benzoic acid (gallic acid)	0.50 \pm 0.01	1.00 \pm 0.03	–	nd	2.00 \pm 0.01	–
17. 4-OH-3-OCH ₃ -benzoic acid (vanillic acid)	0.25 \pm 0.05	1.00 \pm 0.05	–	nd	0.50 \pm 0.01	–
18. 4-OH-3-OCH ₃ -phenylacetic acid (homovanillic acid)	2.00 \pm 0.04	0.50 \pm 0.01	–	nd	1.00 \pm 0.01	–
19. Methyl vanillate	2.00 \pm 0.04	2.00 \pm 0.04	–	1.00 \pm 0.01	1.00 \pm 0.01	–
20. Ethyl vanillate	0.03 \pm 0.01	0.03 \pm 0.01	–	nd	nd	–
21. 4-OH-3,5-di-OCH ₃ -benzoic acid (syringic acid)	0.25 \pm 0.02	0.50 \pm 0.01	–	2.00 \pm 0.07	0.50 \pm 0.01	–
22. Methyl syringate	nd	nd	–	nd	nd	–
Cinnamic acid and its derivatives						
23. Cinnamic acid	0.50 \pm 0.02	0.50 \pm 0.02	–	0.25 \pm 0.01	0.25 \pm 0.01	–
24. 2-OH-cinnamic acid (o-coumaric acid)	2.00 \pm 0.02	2.00 \pm 0.04	–	nd	1.00 \pm 0.01	–
25. 3-OH-cinnamic acid (m-coumaric acid)	0.13 \pm 0.07	0.13 \pm 0.02	2.00	nd	0.25 \pm 0.01	2.00
26. 4-OH-cinnamic acid (p-coumaric acid)	0.13 \pm 0.06	0.13 \pm 0.03	2.00	nd	0.13 \pm 0.08	2.00
27. Methyl p-coumarate	nd	0.50 \pm 0.02	1.00	nd	0.50 \pm 0.02	–
28. 3,4-di-OH-cinnamic acid (caffeic acid)	0.13 \pm 0.03	0.13 \pm 0.04	2.00	0.25 \pm 0.02	0.25 \pm 0.02	–
29. 3,4-di-OCH ₃ -cinnamic acid (caffeic acid dimethyl ether)	0.25 \pm 0.04	0.25 \pm 0.04	2.00	nd	0.25 \pm 0.02	2.00
30. 3,4-di-OH-hydrocinnamic acid (hydrocaffeic acid)	0.50 \pm 0.07	0.50 \pm 0.02	–	nd	1.00 \pm 0.03	–
31. 3-Caffeoylquinic acid (chlorogenic acid)	0.13 \pm 0.02	0.13 \pm 0.03	2.00	nd	0.13 \pm 0.03	2.00
32. 2,3-Dicaffeoyl-L-tartaric acid (chicoric acid)	nd	1.00 \pm 0.04	–	nd	2.00 \pm 0.01	–
33. 3,4-Dihydroxycinnamic acid (<i>R</i>)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (rosmarinic acid)	0.13 \pm 0.03	0.13 \pm 0.02	–	1.00 \pm 0.08	1.00 \pm 0.02	–
34. 4-OH-3-OCH ₃ -cinnamic acid (ferulic acid)	0.25 \pm 0.02	0.25 \pm 0.06	2.00	nd	0.25 \pm 0.01	2.00
35. 3-OH-4-OCH ₃ -cinnamic acid	0.13 \pm 0.03	0.13 \pm 0.01	–	0.25 \pm 0.08	0.25 \pm 0.04	–
36. Methyl ferulate	0.25 \pm 0.10	0.25 \pm 0.01	2.00	0.25 \pm 0.10	0.25 \pm 0.04	2.00
37. Ethyl ferulate	0.13 \pm 0.06	0.13 \pm 0.02	2.00	nd	0.25 \pm 0.01	1.00
38. 4-OH-3,5-di-OCH ₃ -cinnamic acid (sinapic acid)	0.25 \pm 0.04	0.25 \pm 0.05	–	nd	0.25 \pm 0.05	–

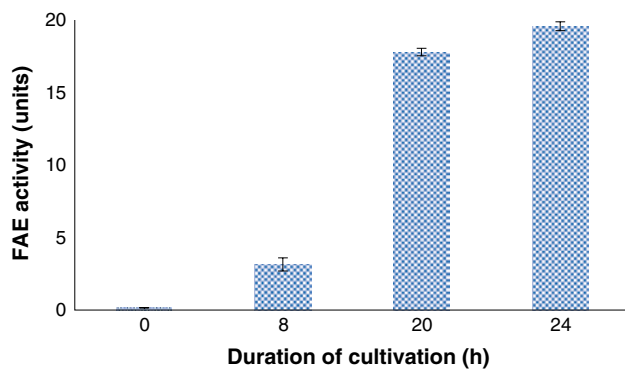


Fig. 1 FAE activity detected in cultured *Lb. helveticus* T159 strain ($n = 2 \pm \text{SD}$)

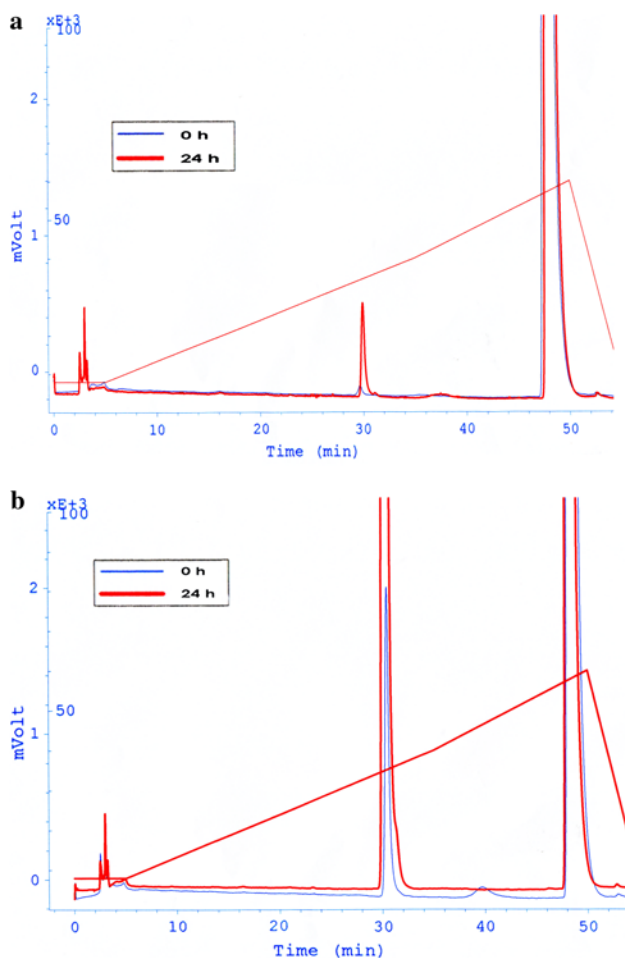


Fig. 2 Free ferulic acid (30.6 min.) released by *Lb. helveticus* T103 (a) and T159 (b). *Thin line*—at the beginning of cultivation (0 h), *bold line*—at the end of cultivation (24 h)

were lower than those for benzoic acid derivatives. After 2 and/or 6 h of cultivations, MIC values for benzoic acid derivatives were higher than those for their corresponding

counterparts: benzoic acid (1) versus cinnamic acid (23), 3-hydroxybenzoic acid (3) versus m-coumaric acid (25), 4-hydroxybenzoic acid (4) versus p-coumaric acid (26), protocatechuic acid (13) versus caffeic acid (28) and veratric acid (14) versus caffeic acid dimethyl ether (29). An exception was the MIC value for salicylic acid (2), which was lower than that for o-coumaric acid (24). The comparison of vanillic acid (17) versus ferulic acid (34) gave unclear results. MIC values estimated for cinnamic acid (23) were significantly lower with regard to *Lb. helveticus* T159 than *Lb. helveticus* T103 cultured for 2 and 6 h. MIC values for o-, m- and p-coumaric acids (24–26) were detected in the case *Lb. helveticus* T159 cultured for 2 h (0.13–2.00 $\mu\text{g}/\text{mL}$), but not in the case of *Lb. helveticus* T103. The comparison of MIC values for ferulic acid (34) and its ethyl ester (37) revealed that this esterification caused a significant decrease in MIC value in the cultured *Lb. helveticus* T159 strain, but no difference was observed in the case of the T103 strain. The strongest antibacterial activity (among cinnamic acid derivatives) was shown by 3-caffeoylquinic acid (31) with an MIC value of 0.13 $\mu\text{g}/\text{mL}$ for both strains. Moreover, in the case of a number of benzoic acid derivatives (2, 4, 8, 10, 11, 14, 17, 19), the MIC value (after 6 h) was higher for *Lb. helveticus* T159 than for T103. Finally, it can be noted that methyl ferulate (36) exhibited a higher antibacterial activity towards *Lb. helveticus* T159 and *Lb. helveticus* T103 than methyl vanillate (19) (in terms of both MIC and MBC values).

Detection of S-layer proteins

Two strains of *Lb. helveticus* were analysed for the production of S-layer proteins. The presence of S-layer proteins was detected in *Lb. helveticus* T159 cells after polyacrylamide gel electrophoresis of whole-cell proteins (Fig. 3).

The cell hydrophobicity assay

SAT and CRB of *Lb. helveticus* T159 and T103 grown on MRS broth are depicted in Fig. 4. A strong correlation was observed between the CRB and SAT results with the S-layer producing the T159 strain, which is the most hydrophobic. Strain T103 demonstrated the same low SAT value, but a more precise quantitative CRB assay implied its less hydrophobic cell surface.

Discussion

Results reported in this study show that the antibacterial activity of benzoic acid derivatives was less effective than that of cinnamic acid derivatives, in terms of the inhibition of the growth of both *Lb. helveticus* strains. This result can

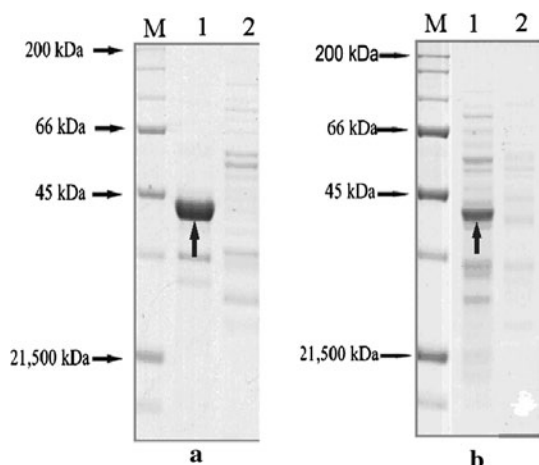


Fig. 3 SDS-PAGE profile of whole-cell proteins (**a**) and cell surface proteins extracted with 5 M LiCl (**b**) from *Lactobacillus helveticus* strains. Lane: M: protein ladder MW marker; 1: *Lb. helveticus* T159; 2: *Lb. helveticus* T103. S-layer proteins are indicated by arrows

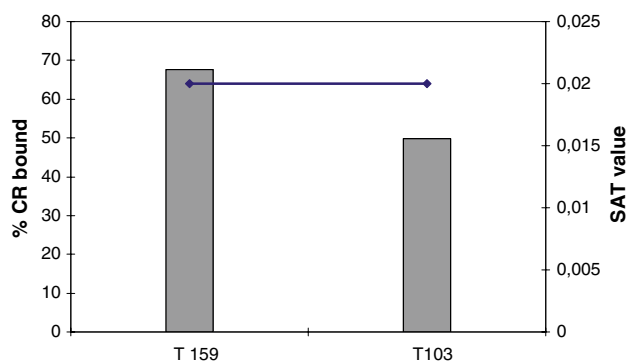


Fig. 4 CRB (bars) and SAT (points) of *Lb. helveticus* T159 and T103 strains. Arithmetic mean and standard error (± 0.02) are from three experiments

be explained by the presence of the longer hydrophobic propenoic side chain in cinnamic acid and its derivatives, in comparison with the short carboxylic group attached to the phenol ring of benzoic acids (higher lipophilicity of cinnamic acid derivatives). However, benzoic acid, and not cinnamic acid, is a well-known antibacterial agent used in the food industry. Taking under consideration MIC and MBC values of compounds 1–22, it seems that *Lb. helveticus* T159 was, in many cases, more sensitive to benzoic acid and its derivatives than *Lb. helveticus* T103 strain. Based on the results of the FAE activity in *Lb. helveticus* T159 (Fig. 1), it can be assumed that FAE is constitutively synthesized at low levels in the cell. The inhibitory effect, at least partly, can be therefore attributed to the effect of FAE activity leading to the release of a free phenolic acid, a more toxic product. Although the activity of FAE towards the esters of benzoic acid is not routinely pointed out, low

activities of FAE produced by *Lb. acidophilus* K1 were previously detected in the presence of methyl vanillate or methyl syringate [6]. Moreover, the growth of *Lb. helveticus* T159 in the presence of various esters of phenolic acids can be considered in terms of the substrate specificity of FAE. Lai et al. [16] reported that FAE from *Lb. johnsonii* showed a high affinity towards aromatic esters, especially ethyl ferulate. In our study, FAE activity in *Lb. helveticus* T159 was not detected in the presence of methyl vanillate which is also aromatic compound, but it is lacking the additional aliphatic chain, in comparison with methyl esters of cinnamic acids.

Within the frame of this study, an attempt was made to establish a coherent relationship between the molecular structures of test compounds and their antibacterial activities in model systems containing one compound at a time. Previously, Sánchez-Maldonado et al. [17] presented a short review on the antimicrobial activity of free phenolic acids towards *Lactobacillus* strains. These authors tested (singly) the ability of 6 hydroxybenzoic and 6 hydroxycinnamic acids to inhibit the growth of *Lb. plantarum* and *Lb. hammesii*. The antimicrobial activity of hydroxycinnamic acids was comparable or higher than that of hydroxybenzoic acids that contain the same number of OH hydroxyl groups. Next, the role of the number and position of OH and OCH₃ groups should be analysed within the scope of the aims of our study. Our results suggest that the *meta*- and *para*-positions of a single OH group is neutral for the antibacterial activity because the MIC values of benzoic acid (1) and its derivatives, 3- and 4-hydroxybenzoic acids (3,4), were similar. However, the *ortho*-position of the OH group (salicylic acid, 2) significantly increased the antibacterial activity (both MIC and MBC). The role of the OCH₃ group could probably be negligible because no substantial difference in MIC was observed between ferulic acid (34) and its relative compound (35) with conversely substituted OH and OCH₃ groups. Other authors [17] showed that the antibacterial activity of phenolic acids decreased with the increased number of OH groups in hydroxybenzoic acids, whereas it had a minor effect on the activity of hydroxycinnamic acids. Moreover, the substitution of the OH group with the OCH₃ group enhanced the antibacterial activity of hydroxybenzoic acids, but it had no effect on the activity of hydroxycinnamic acids. In our work, the results generally are in agreement with the observations published in the cited works. However, the interpretation of these results is hard, due to many factors influencing the growth-inhibitory activity of phenolic acids and their derivatives on bacteria in our model systems. For example, it cannot be clearly resolved if methyl and/or ethyl esters of vanillic, 4-hydroxybenzoic and ferulic acid, were more efficient growth inhibitors than corresponding free phenolic acids (Table 1). Two factors should be considered for an analysis

of these observations. First, the hydrophobicity of phenolic acid esters is higher than the hydrophobicity of corresponding free compounds, due to the esterification of the carboxyl group. Therefore, the migration of the ester into the surface layers of the bacterial cells that contain hydrophobic S-proteins (T159 but not T103) will be improved in comparison with the free phenolic acid. It is most probably that the lipophilicity of the compound (the substitution of the benzene ring by OH and OCH₃ groups) plays an important role in this context. The second factor that should be considered is the presence of FAE activity in the supernatant and the cell wall, as well as in the cytoplasm (in the case of T159; detailed results to be published soon). Once the ester is hydrolysed, free phenolic acid appears in cell membranes and intracellularly and, therefore, can exert its growth-inhibitory activity. The role of pK_a of individual phenolic acids should be mentioned in this context. This hypothesis proves that the model presented in our study is very complex, due to the simultaneous presence of FAE and free and esterified phenolic acid in the environment, cell membranes and the cytoplasm of the bacteria. In our model, these two factors are at variance with each other, because while the enzyme decreases the penetration of the test compounds via hydrolysis of the esters, the more hydrophobic esters are transformed to less hydrophobic free compounds. The antibacterial action of free, non-dissociated phenolic acids is higher than their corresponding esters due to the release of hydrogen cations inside the cell. Therefore, the proportion of free and esterified phenolic acid is changed in time, and the interpretation of results is very difficult.

Last but not least, the possible role of S-layer proteins should be taken under consideration. The surface properties of bacteria determined its interactions with the environment, particularly non-specific interactions governed by the physicochemical properties of the outer constituents of the bacterial cell wall [18]. The cell wall of *Lb. helveticus* consists mainly of peptidoglycans, teichoic acids, polysaccharides and proteins. The surface proteins in many strains of *Lb. helveticus* are S-layer proteins. These are expected to have appreciable effects on the properties of the cell walls of bacteria, because of their highly basic nature and isoelectric point above pH = 9 [19]. *Lb. helveticus* T159 showed the presence of specific hydrophobic cell surface proteins, such as the S-layer. There is some information about the protective role of S-layer proteins; e.g. their negatively charged residues can bind toxic ions [20]. Our study did not confirm the protective role towards phenolic acids of the S-layer proteins in *Lb. helveticus* T159. Moreover, it should be underlined that S-layer proteins are strongly hydrophobic [18] and phenolic compounds should demonstrate the ability to strongly diffuse into this

layer. Therefore, the higher sensitivity of *Lb. helveticus* T159 strain in comparison with *Lb. helveticus* T103 can be, at least partly, due to the difference in the composition of the cell surface (hydrophobicity). It is likely that hydrophobic interactions between membrane lipids and phenolic compounds and their metabolites are involved in cell damage. The mechanisms by which phenolic acids inhibit bacterial growth are not well known. García-Ruiz et al. [21] reported that phenolics damaged the bacteria cell membrane causing alteration in transport, energy-dependent processes and metabolic pathways that are essential for bacterial viability.

Lee et al. [22] reported that intestinal bacteria metabolize phenolics and produce different aromatic metabolites which can be retained by the bacterial cell and this bacterial activity can explain the effects on the growth. Also, metabolites released into the media may influence the growth of bacteria that produce these metabolites. The ability to synthesize FAE by intestinal microflora is the first step in the extensive biotransformation of phenolic compounds in the intestine leading to an increase in their intestinal adsorption and bioavailability. A thorough discussion of the characteristics and biological role of FAEs can be found in the review by Lai et al. [23]. Also, Sánchez-Maldonado et al. [17] detected the decarboxylation and/or reduction in phenolic acids (chlorogenic, caffeic, p-coumaric, ferulic and protocatechuic acids) by *Lb. plantarum*, *Lb. fermentum*, *Lb. reuteri* and *Lb. hammesii*. These biotransformations caused the two- to fivefold lower antibacterial activity in comparison with initial substrates. This result explains the important role of the detoxification of phenolic compounds by lactic acid bacteria.

In the light of the results obtained within this survey, it can be concluded that the growth-inhibitory activity of phenolic acids and their derivatives towards both bacterial strains strongly depended on the presence of FAE in the bacterial environment. A significant role, in our opinion, can be also attributed to the composition of the surface of bacterial cells (the presence of S-proteins). The molecular composition of free phenolic acids and their derivatives (the number and/or position of OH or CH₃) seems to play a minor role in this context. Further studies on this subject are in progress.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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