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Impact of antimicrobials, naturally produced by lactic acid bacteria, on the *Listeria monocytogenes* growth in minced salmon

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Abstract: The effect of antimicrobials produced by lactic acid bacteria (LAB) on the inhibition of *Listeria monocytogenes* ATCC 19111 in minced salmon was analysed and compared to the sodium lactate and bacteriophage action during storage at 6 °C. All tested additives showed a quite noticeable reduction of *L. monocytogenes* counts by 30–95% compared with control samples. Antimicrobials produced by the tested *Enterococcus faecium* strains showed moderate inhibitory activity while the greatest inhibitory activity was observed for antimicrobials produced by *Streptococcus thermophilus* 43 directly in the same way as the additive sodium lactate. The correlation was determined within inhibitory efficiency and produced total fatty acid amounts. *S. thermophilus* 43 showed the exceptionally stronger inhibition index for *L. monocytogenes* and yielded the higher monounsaturated fatty acid amount (42%) than *E. faecium* strains. Both *E. faecium* strains showing the lower inhibition efficiency produced the highest polyunsaturated fatty acid amounts (21.7–29.5%). *E. faecium* L41-2B-2v and *S. thermophilus* 43 were found to produce bioactive compounds like omega-3 and omega-6 FAs.

Keywords: antimicrobic action; probiotics minced salmon; antilisterial action; fatty acids; model food system

In terms of food safety, fish is a perishable food product because its chemical composition and its structure are favourable for the growth of microbiota. Pathogenic microbiota is a key risk factor that needs to be evaluated and eliminated in the production of fishery products. *Listeria monocytogenes* is one of the most dangerous pathogenic species found in fish. Listeriosis caused by *L. monocytogenes* is a potentially fatal disease, particularly dangerous to people with the weakened immune system, pregnant women, and children. Thus, special attention should be given to the control and prevention of these bacteria in fish products (Ress et al. 2017). Some fish production processes lack a listericidal processing operation, whereas the parameters of freezing, curing and smoking processes fail to ensure complete inactivity of *L. monocytogenes* (Porsby et al. 2008). Therefore, it is currently extremely difficult to produce some fish products where *L. monocytogenes* is absent, since the production process is not efficient enough in terms of listericidal processes. These bacteria are extremely viable and can grow in a production environment and re-infect the final product (Branciari et al. 2016). Given that it is impossible to completely inactivate *L. monocytogenes* during fish production processes, it is necessary to control their growth in the product. The risk of listeriosis is likely to be very low if the product contains less than 100 CFU g^{-1} of *L. monocytogenes*. Contamination can also occur on the surface of the product during post-process exposure and steps such as peeling and packaging are potential routes for pathogen entry (Vijayakumar & Muriana 2017). The use of additives to prevent food spoilage is not favoured by the public and it is carefully controlled by law. The highlighted aspect in food preservation is the use of antimicro-

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bials naturally produced by lactic acid bacteria LAB (Kamiloglu et al. 2019). LAB could exert a strong antimicrobial activity against many kinds of microbiota, including food spoilage microorganisms and pathogens. According to a previous study (Šalomskienė et al. 2019), *E. faecium* and *S. thermophilus*, these LAB strains with antimicrobial potential, have been identified. These LAB strains were chosen for further investigations to evaluate their potential to inactivate and/or control the growth of *L. monocytogenes* in a model food system and compare the inhibitory activity with the chemical additive sodium lactate and bacteriophage. The research also provides a characterisation of antimicrobial composition by the presence of fatty acids as a functional feature for the quite noticeable inhibition effect on *L. monocytogenes* by tested bacteria.

MATERIAL AND METHODS

Preparation of model food system. The model food system (MFS) was created by using the minced raw salmon (MRS) purchased in a local supermarket. The product free from *L. monocytogenes* was used for experiments and contaminated by a suspension of the reference strain *L. monocytogenes* ATCC 19111 to reach the cell concentration of 10^3 CFU g^{-1} and/or 10^6 CFU g^{-1}. The minced raw salmon was well mixed, so the suspension of the strain was spread out evenly throughout the mass. It was divided into 100 g samples and each sample was placed into a sterile container. Some of the samples were used as control samples. To analyse an inhibitory effect on *L. monocytogenes* ATCC 19111, different additives: 2.5 mL cell-free supernatant (CFS) produced by tested lactic acid bacteria (LAB); sodium lactate 3%, and bacteriophage solutions 50, 100 and 200 µL were added into separate samples and mixed to a uniform concentration. Samples were stored at 6 °C. The reagents, sodium lactate (PURASAL S) and bacteriophage solution (Listex P100) used in the study were of analytical grade and procured from EBI Food Safety (Netherlands).

Bacterial strains and growth conditions. The count of *L. monocytogenes* in the MRS was determined on Listeria Agar according to Ottaviani and Agosti (ALOA, Liofilchem Diagnostici, Italy) according to ISO 11290-2:2017 (2017, Microbiology of food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. – Part 2: Enumeration method). The foodborne *L. monocytogenes* strains used in this study were isolated from the raw fish samples. Isolated cultures and reference culture of *L. monocytogenes* ATCC 19111 were stored on PCA (Plate Count Agar) slant agar at 5 °C and reinoculated before testing. Target cultures were washed with a sterile physiological solution, and the densities of cell suspensions were adjusted according to a 0.5 McFarland standard. *S. thermophilus* 43, *E. faecium* L59-30 and L41-2B-2v (a microbiological culture collection, the Food Institute, Kaunas University of Technology, Kaunas, Lithuania) were cultivated in sterile milk at 37 °C for 24 h. One millilitre of the culture was added in 10 mL of a modified mMRS broth (Merck KGaA, Germany) and incubated at 37 °C for 48 h and/or 72 h (all three strains). CFS was obtained by removing the cells by centrifugation at 6 000 × g for 15 min followed by filtering through 0.22 µm pore filter (Merck KGaA, Germany). Obtained CFS was added into the MFS samples for performing a microbiological analysis.

The agar well diffusion method was used to determine the antibacterial activity of the LAB strains. After incubation of all three LAB strains at 37 °C for 48 and 72 h, the plates were observed for the zones of growth inhibition around the wells. A cell suspension mixture of 10 foodborne *L. monocytogenes* strains from smoked fish products was used for testing the antimicrobial activity of CFS.

Fatty acid analysis. Fatty acids (FAs) were separated using a gas chromatograph (Shimadzu GC-2010; Shimadzu, Japan) equipped with flame ionisation detector according to the conditions described by Jonkuvienė et al. (2014).

Statistical analysis. The chemical and microbiological tests were repeated three times. For microbiological tests, a repetition plot design was used with tested material on the main plot versus the reference *L. monocytogenes* ATCC 19111 strain on the subplot, which was then split into storage conditions. The significant effect of inhibition was evaluated by the inhibition index defined as follows:

$$\text{Inhibition index (II)} = 1 - \frac{\log\left(\frac{N}{N_o}\right)\text{treated}}{\log\left(\frac{N}{N_o}\right)\text{untreated}}$$

where: N – number of cells (CFU g^{-1}) at the end of the storage time; N_o – number of cells (CFU g^{-1}) at the beginning of the storage; log (N/N_o) of the treated and untreated (control) samples was evaluated during storage (Giannuzzi & Zaritzky 1993).

Data were analysed using Excel software 11 (Microsoft, USA), standard deviations (SD) and *t*-tests were calculated and referred to as the statistically

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significant difference with $P < 0.05$ between the obtained research results.

Scanning electron microscopy (SEM). *L. monocytogenes* ATCC 19111 strain grown on PCA agar at 37 °C for 24 h was used for the preparation of 10^6 CFU mL^{-1} suspension and mixed with CFS obtained from *S. thermophilus* 43 (1 : 1 v/v). The cells were exposed to 6 °C for 72 h, then the mixture was centrifuged at 3 000 × *g* for 30 min and the cells were washed with sterile water three times. The cells were placed onto silicon plates, dried in the air, and assessed under SEM (S-3400 N; Hitachi, Japan) operating at 5 kV.

RESULTS AND DISCUSSION

The ability of food to support the growth of *L. monocytogenes* increases the risk to food safety. Many factors need to be considered in determining whether the microbial growth is inhibited to a required level before the food is consumed.

In a previous study (Šalomskienė et al. 2019), two *E. faecium* strains were found with genes cloning enterocins A and P, while *S. thermophilus* 43 was found to produce a high amount of proteins. Natural antimicrobials produced by bacteriocinogenic and proteinaceous LAB were leading candidates for practical application of selected strains in the MFS.

In order to specify the antimicrobial efficacy of CFS depending on the incubation time of tested cultures, antimicrobial properties were verified against foodborne strains and the reference strain *L. monocytogenes* ATCC 19111 on agar plates. The tested CFS had a significantly higher antibacterial activity against the reference strain in direct contrast to foodborne *L. monocytogenes* strains. CFS showed a higher activity when LAB strains were incubated for 3 days compared with 2 days (Table 1). For that reason, further investigations were carried out using CFS prepared after 3 days of LAB incubation in order to obtain the highest inhibition applicable to *L. monocytogenes* in the MFS.

The ability of antimicrobials within CFS to inactivate and/or control the growth of *L. monocytogenes* during food storage and its comparison with the chemical additives sodium lactate (SL) and bacteriophage were evaluated. There is a specified limit of 100 CFU g^{-1} for foods in which the growth of *L. monocytogenes* will not occur. For that reason, the MFS was contaminated by a concentration of 10^3 CFU g^{-1} of the *L. monocytogenes* inoculum. The initial inoculum of *L. monocytogenes* was decreased by 70% using CFS produced from *S. thermophilus* 43 and by 30% using both tested CFS produced from *E. faecium* cultures (Figure 1A).

CFS were not able to inhibit *L. monocytogenes* ATCC 19111 in the MFS during storage at 25 °C while the presence of the tested additive in the MFS showed a significantly different growth pattern compared to control samples. During the 24 h storage period the count of *L. monocytogenes* increased around twice in all tested variants but during storage for another 48 h CFS produced an effective inhibition by decreasing the *L. monocytogenes* count contrary to control samples where the *L. monocytogenes* count was still slightly increased (Figure 1B). Similarly, the growth of *L. innocua* inoculated onto fresh-cut onions was re-

Table 1. Antimicrobial activity of the tested CFS against the target *L. monocytogenes* strains (mean ± SD)

Bacterial strain	Zone of inhibition (mm)			
	L. monocytogenes ATCC 19111*	Foodborne *L. monocytogenes**	*L. monocytogenes* ATCC 19111**	Foodborne *L. monocytogenes***
***S. thermophilus* 43** Live cells	20.0 ± 0.2	13.5 ± 1.2	25.0 ± 0.2	17.8 ± 1.6
CFS	20.0 ± 0.0	16.0 ± 1.0	25.0 ± 0.0	21.5 ± 1.2
***E. faecium* L59-30** Live cells	18.3 ± 0.2 b	13.5 ± 0.8	27.0 ± 0.1	19.6 ± 0.6
CFS	15.2 ± 0.1 b	14.0 ± 0.8	20.0 ± 0.3	19.5 ± 0.8
***E. faecium* L41-2B-2v** Live cells	18.5 ± 0.0	14.0 ± 1.0	27.0 ± 0.0	19.1 ± 0.9
CFS	16.0 ± 0.2	12.5 ± 1.0	21.0 ± 0.0	19.3 ± 0.9

CFS – cell-free supernatant; *CFS prepared after 2 days of LAB (lactic acid bacteria) incubation; **CFS prepared after 3 days of LAB incubation; SD – standard deviation

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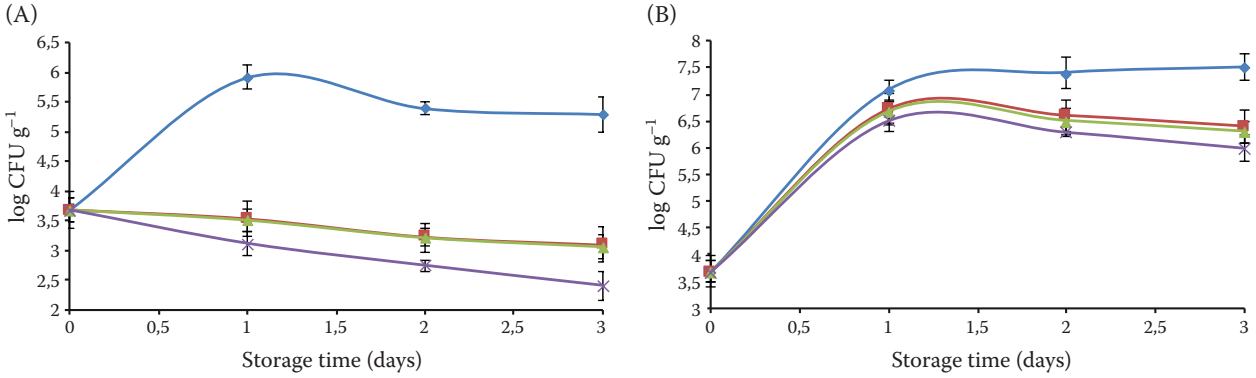

Figure 1. *L. monocytogenes* ATCC 19111 inhibition kinetics in the MFS under storage at 6 °C (A) and 25°C (B)

Blue line – control samples; experimental samples: orange line – samples treated with CFS of *E. faecium* L59-30; green line – L41-2D-2V; violet line – samples treated with CFS of *S. thermophilus* 43; MFS – model food system; CFS – cell-free supernatant

duced by 1.0 to 1.6 log CFU g^{-1} and significantly inhibited after 12 days of storage at 5 °C due to the presence of LAB (Yang et al. 2012). Vijayakumar & Muriana (2017) examined the effectiveness of LAB to prevent the growth of *L. monocytogenes* on hotdogs and found a > 2 log decrease of *L. monocytogenes* in treated samples and 6–7 log difference vs. controls. Martinez et al. (2015) found that the growth of *L. monocytogenes* was inhibited at 4 °C and 15 °C for up to 28 days, especially *L. monocytogenes* 4b in the food product kept at 15 °C due to the production of bacteriocins. The results obtained in this study revealed that the effectiveness of LAB inhibition on target food pathogens could depend on the LAB strain used.

Sodium lactate is used in meat products as a humectant, flavour enhancer, and antimicrobial agent in meat and poultry products (Shelef 1994), as well as a shelf life extender as a replacement for sodium chloride. This resulted in a less salty taste (Houtsma et al. 1996). Slower inhibition was seen in samples with a higher *L. monocytogenes* inoculum size of 10^6 CFU g^{-1} in the MFS during 5 days of storage, while the inhibition effect of SL did not differ between low (10^3 CFU g^{-1}) and high initial inoculum sizes at the end of 10 days of storage. The counts decreased by 28 and 22 %, respectively (Figure 2).

The concentration of 10^6 CFU g^{-1} *L. monocytogenes* counts in control samples decreased in a similar pattern to that of using the additive SL but 2-log cycles more slowly at the end of storage. SL in the presence of the low 10^3 CFU g^{-1} *Listeria* spp. contamination suppressed the growth of tested bacteria during the entire experiment. Hwang et al. (2012) reported that lactate at 3% may prevent the growth of *Listeria* spp. in ham at refrigerated temperatures for up to 35 days, while lactate at lower levels slows the growth of these pathogens. Results obtained in this research confirm the effectiveness of inhibition by SL at 3%, but also it should be noted that the decreasing rate of *Listeria* spp. counts depended on the inoculum size.

The addition of bacteriophage to the MFS was quite effective for *L. monocytogenes* ATCC 19111 inhibition and during 2 h of treatment it allowed a significant decrease of counts by more than 95% (Figure 3). Phages are excellent as food biopreservation agents since they are reported to lyse their hosts at temperatures as low as 1 °C (Greer 1988) limiting the growth of pathogenic and spoilage bacteria on even refrigerated foods. The use of a phage for *Listeria* spp. inhibition is provided in food

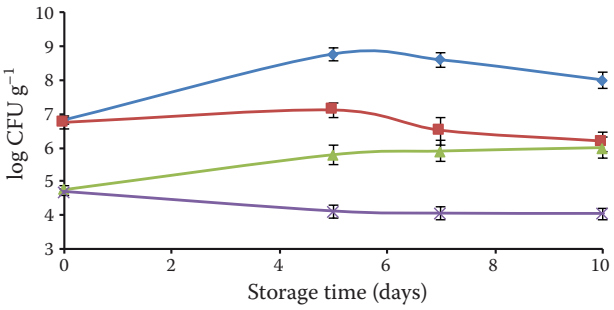

Figure 2. *L. monocytogenes* ATCC 19111 inhibition kinetics in the MFS under storage at 6 °C in the presence of sodium lactate 3%

Blue line – control samples; experimental samples: red line – with the inoculum 10^6 CFU g^{-1} of *L. monocytogenes*; green line – control samples; experimental samples: violet line – with the inoculum 10^3 CFU g^{-1} of *L. monocytogenes*; MFS – model food system

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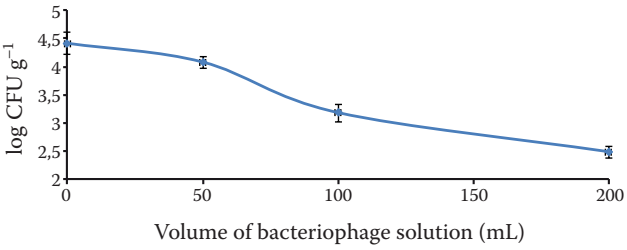

Figure 3. *L. monocytogenes* ATCC 19111 inhibition kinetics in the MFS in the presence of a bacteriophage

MFS – model food system

models such as the surface of poultry meat (Bigot et al. 2011) and fresh fish fillets (Soni et al. 2010).

Predictive models play an important role, along with other supporting information, in determining if a given product formulation or process will reduce the likelihood of *Listeria* spp. presence or growth. In order to evaluate a statistically reliable effect of additives tested for the reference *L. monocytogenes* growth suppression, the inhibition indices (II) were calculated according to Equation 1 using the experimental data obtained from Figures 1–3. Linear correlations for the inhibition of tested additives during storage were computed and *R*-squared values higher than 0.9 were obtained (Figure 4). The highest degree of inhibition by CFS from *S. thermophilus* 43 was evaluated. The treatment with high inoculum with the additive SL acted bactericidally after 7 days of storage (II < 1) while the treatment with low inoculum acted strongly bactericidally (II > 1.5). Results showed that CFS from LAB could be used as a strong bactericidal agent to control the *L. monocytogenes* ATCC 19111 growth in the same way as the additive SL.

The SEM micrograph showed the smooth surface of *L. monocytogenes* ATCC 19111 untreated cells, while cells exposed to CFS produced by *S. thermophilus* 43 were damaged (Figure 5) and confirmed the antilisterial action.

The tested LAB cultures were found to produce antimicrobials such as ethanol, lactic acid, citric acid and the produced amounts of these substances did not significantly differ depending on the strain (Šalomskienė et al. 2019). This study was focused on the characterisation of different compounds produced within CFS of tested LAB such as fatty acids (FAs)

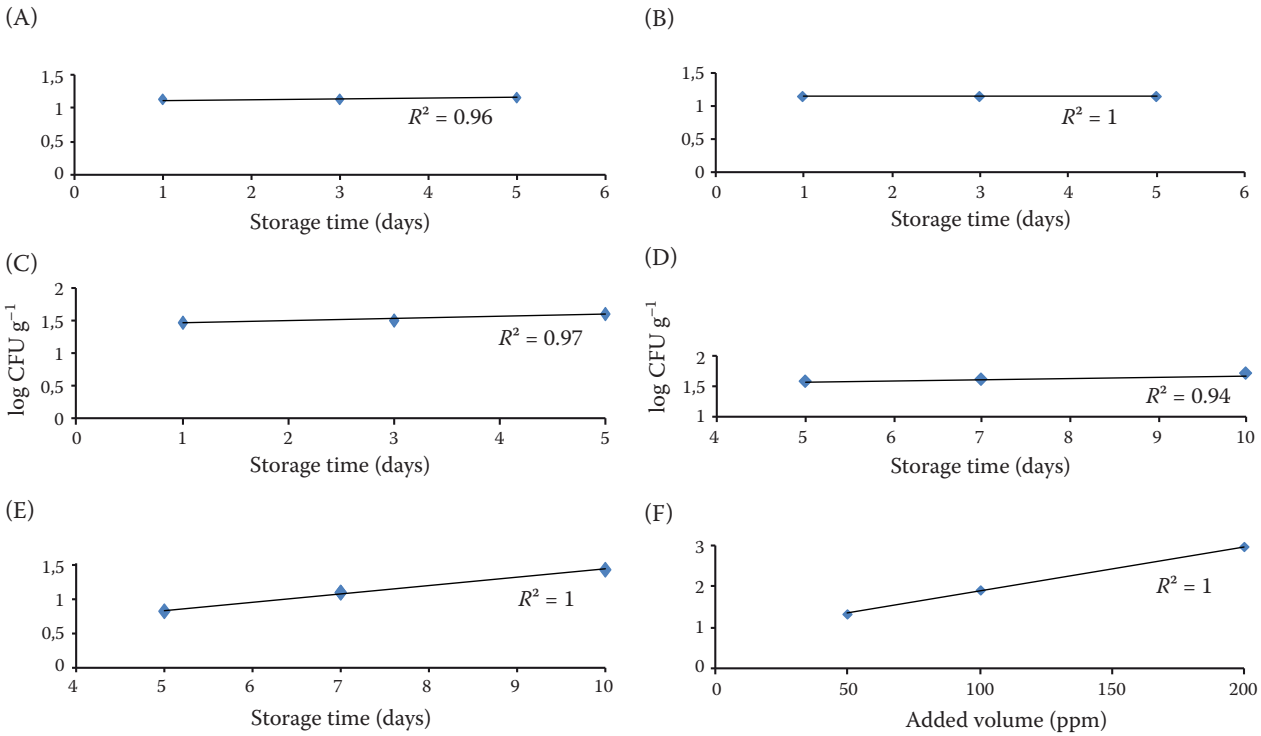

Figure 4. Inhibition index plot design with the tested material (CFS) versus the storage time in days (A–E) and added volume (F): *E. faecium* L59 (A); *E. faecium* L41-2B-2v (B); *S. thermophilus* 43 (C); sodium lactate vs. 10^3 CFU g^{-1} (D); 10^6 CFU g^{-1} of inoculum (E); bacteriophage (F)

CFS – cell-free supernatant

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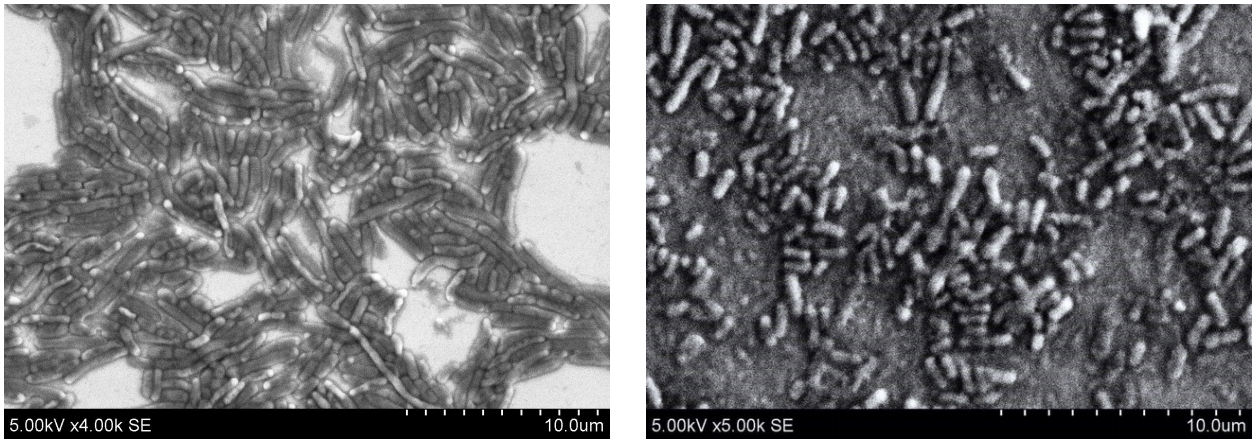

Figure 5. Scanning electron microscopy images of *L. monocytogenes* ATCC 19111 cells (A) and cells exposed to CFS of *S. thermophilus* 43 (B)

CFS – cell-free supernatant

to Figure out their potentials for giving the antimicrobial activity to *L. monocytogenes*. It was found that all tested LAB strains produced different total FA (Table 2) amounts while the total FA composition pattern was similar in both *E. faecium* strains. CFS prepared after 3 days of LAB incubation in mMRS broth had a higher antimicrobial activity than CFS prepared after LAB incubation for 2 days (Table 1). Differences in the total FA composition were seen within tested LAB and some determined FA amounts correlated with antimicrobial activity. Higher amounts of polyunsaturated FAs were found within *E. faecium* while higher saturated FA amounts were found within *S. thermophilus* 43 after 3 days of incubation.

The tested LAB after 2 days of incubation produced FAs such as tetradecane, butane, pentane, hexane, heptane, octane, nonane, decane, cis-9-decene, undecane, dodecane, tridecane, pentadecane, cis-10-pentadecane, hexadecane, palmitolein, heptadecane, cis-10-heptadecene, octadecane, olein, linol, eicosane, α-linolene, γ-linolene, docosatetraen, and docosane. The longer incubation time resulted in only cis-10-heptadecene production within *E. faecium* L59-30 and cis-11,14,17-eicosatriene production within *S. thermophilus* 43. Using highly antimicrobially active LAB strains, producing significant amounts of FAs as an additive to food products, could be not only safe but also healthy.

CONCLUSION

The effects of naturally produced antimicrobials by selected LAB on the inhibition of *L. monocytogenes* depended upon the LAB strain used. *S. thermophilus* 43 had the best characteristic showing good antimicrobial activity *in vitro* as well as high interference activity with *L. monocytogenes* contaminants *in vivo*. Comparison of inhibitory activity within natural antimicrobials, sodium lactate and the bacteriophage confirmed an effective action of natural antimicrobials equivalent to sodium lactate at the concentration

Table 2. The total amounts of fatty acids produced within the tested CFS from LAB strains (mean ± SD)

LAB	LAB incubation time (h)	Fatty acid from the total fatty acid amount (%)					
		saturated	monoun-saturated	polyun-saturated	trans	omega-3	omega-6
E. faecium L59-30	24	57.8 ± 2.0	35.8 ± 2.1	6.4 ± 0.4	–	0.5 ± 0.1	5.4 ± 1.0
	72	48.2 ± 1.4	30.1 ± 1.4	21.7 ± 1.2	–	–	–
E. faecium L41-2B-2v	24	54.1 ± 1.0	37.8 ± 1.5	8.1 ± 0.4	–	0.5 ± 0.1	7.6 ± 1.0
	72	50.8 ± 0.8	19.7 ± 0.8	29.5 ± 1.8	–	8.6 ± 0.5	17.8 ± 1.1
S. thermophilus 43	24	28.7 ± 1.2	48.8 ± 2.4	22.4 ± 1.9	0.2 ± 0.1a	0.3 ± 0.1	22.0 ± 3.0
	72	47.8 ± 1.2	42.4 ± 1.8	9.4 ± 1.0	0.4 ± 0.1a	1.7 ± 0.4	7.7 ± 0.4

CFS – cell-free supernatant; LAB – lactic acid bacteria; (–) LAB did not produce these fatty acids

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of 3%. Lactic acid bacteria having strong antimicrobial activity against food pathogens could be used in ensuring the health safety of food systems and at the same time supplementing it with bioactive compounds such as omega-3 and omega-6 fatty acids.

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