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Effect of *Lactobacillus spp.* strains on the population of *Listeria monocytogenes* isolated from the human vagina

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Medical Research Journal 2021;
Volume 6, Number 1, 8–15
10.5603/MRJ.a2021.0001

ISSN 2451-2591
e-ISSN 2451-4101

ABSTRACT

Introduction: The normal vaginal microbiota (mainly *Lactobacillus* spp.) affects the health of these areas. Bacterial vaginosis is a serious health problem among many women, especially dangerous for pregnant women. The study aimed to assess the impact of *Lactobacillus* spp. strains on the population of *Listeria monocytogenes* isolated from women.

Materials and methods: The research material consisted of reference strains of *Lactobacillus* spp.: *L. acidophilus* (LAC), *L. fermentum* (LFE), *L. gasseri* (LGA), *L. plantarum* (LPL), the strain *L. monocytogenes* ATCC 19111 and 7 *L. monocytogenes* strains isolated from the vagina.

Results: The highest antagonistic activity was shown for the mixed culture of all *Lactobacillus* strains (LACTO MIX) used in the experiment. Among the individual strains of *Lactobacillus* spp. strains, *L. plantarum* turned out to most effectively reduce *L. monocytogenes* number (reduction of 5.74 log CFU $\times$ ml^{-1}). The least effective in inhibiting the growth of *L. monocytogenes* was the *L. acidophilus* strain (reduction of *L. monocytogenes* of a number of 2.21 log CFU $\times$ ml^{-1}).

Conclusions: The presence of *Lactobacillus* spp. in the genital tract limits the development of bacterial infections, which is an important aspect especially for pregnant women.

Key words: *Lactobacillus* spp., *Listeria monocytogenes*, vaginal disease, probiotics, antagonistic action

Med Res J 2021; 6 (1): 8–15

Introduction

The microbiological profile of the vagina can form a stable ecosystem that contributes to maintaining vaginal health, preventing and eliminating the risk of infection. Disturbance of the right amount of bacterial microbiota promotes the development of bacterial vaginosis [1]. The condition of the vaginal microbiota depends on several factors, including age, health, eating habits, endocrine system and hygiene. The composition of the vaginal microbiome of women varies, depending on the part of the world [2–4]. Normal vaginal pH of premenopausal women may range from 3.5 to 4.5, as a result of the presence of different *Lactobacillus* spp. (10^7–10^8 CFU/g vaginal mucus in healthy premenopausal women), i.e. *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. iners*, *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. vaginalis*, *L. delbrueckii*, *L. salivarius*, *L. reuteri*, *L. rhamnosus* [5]. These bacteria are capable of producing lactic acid from glycogen and constitute from 80% to 95% of the vaginal microbiota of healthy women [6]. The vaginal vault is colonized within 24 hours of the birth of the girl, and the process continues until death [5]. Lactobacilli produce hydrogen peroxide, which limits vaginal colonization by catalase-negative bacteria and anaerobes. These products also affect the ability to adhere and compete for adhesion sites in the vagina with pathogenic microorganisms [1]. An important feature of the genus *Lactobacillus* is the synthesis of antimicrobials, which they can produce under aerobic and anaerobic conditions, such as peptides, bacteriocins and biosurfactants, which promote the xenophagy (absorption and degradation by the host cells) of bacteria, viruses and protozoa. Thus, the positive role of lactobacilli is based on the inhibition of growth of other potentially pathogenic endogenous bacteria and prevention of the infection by exogenous bacteria. Therefore, the domination of *Lactobacillus* spp. in the vagina is essential for maintaining the women's health [7].

One of the most dangerous pathogens for pregnant women is *Listeria monocytogenes*. Pregnant women are 18 times more likely to be infected than in the general population [1]. While the maternal disease is usually mild, it can be severe and potentially fatal in newborn babies [5]. It is believed that about 5–10% of women are asymptomatic carriers of *L. monocytogenes* in the vagina and within the gastrointestinal tract [8]. An increase of the vaginal pH enables multiplication of *L. monocytogenes*, thereby posing a risk of the pathogen transmission from the mother to foetus/newborn via placental barrier or the birth canal [9]. Listeriosis most often occurs in the third trimester of pregnancy (from 28 weeks) and is rarely fatal for the mother, especially in the absence of concomitant diseases [10]. Symptoms of neonatal listeriosis include bacteraemia, respiratory failure, purulent conjunctivitis and skin lesions. The estimated incidence of pregnancy-related listeriosis ranges from 1 to 25 cases per 100 000 births, accounting for up to 35% of all *L. monocytogenes* infections [9]. The frequency of neonatal listeriosis is approximately 8.6/100 000 live births, with high mortality (20–60%) and is one of the most common causes of neonatal meningitis [9].

The study aimed to assess the impact of *Lactobacillus* spp. strains on the population of *L. monocytogenes* isolated from the female vagina.

Materials and methods

Bacterial strains

Four *Lactobacillus* spp. reference strains were used in the study: *L. acidophilus* ATCC 314 (LAC), *L. fermentum* ATCC 9338 (LFE), *L. gasseri* ATCC 19992 (LGA) and *L. plantarum* ATCC 8014 (LPL), the reference strain *L. monocytogenes* ATCC 19111 and 7 *L. monocytogenes* strains of serotype 1/2a-3a isolated from the vagina of healthy women. Clinical strains used in the study come from the collection of the Department of Microbiology, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland.

Assessment of the number of *Lactobacillus spp.* in cultures without *L. monocytogenes* and in mixed cultures with *L. monocytogenes* strains

Lactobacillus spp. strains were plated on Rogosa Agar (Merck) and incubated at 35°C (72 h, microaerophilic conditions). For each strain, 10 suspensions of single colonies in LAPTg medium (5 ml of 0.5 McF) were prepared (medium composition: Pepton Tryptone 10 g/l, yeast extract 10 g/l, Pepton 15 g/l, glucose 10 g/l, Tween 80 1 ml/l) (Merck). *L. monocytogenes* strains were plated on Columbia Agar medium with 5% sheep blood (BioMerieux). After 24 h at 37°C single colonies were used to make suspensions in LAPTg medium (5 ml of 0.5 McF). Then mixed cultures were prepared with the following composition: LAC + LMO (each tested strain separately), LFE + LMO (each tested strain separately), LGA + LMO (each tested strain separately) and LPL + LMO (each tested strain separately) and mix of all tested *Lactobacillus* spp. strains (LACTO MIX). The volume of each bacterial suspension was 5 ml. The negative control consisted of mixtures: the reference strain *Lactobacillus* spp. + 5 ml of sterile LAPTg medium, the mixture of reference strains of *Lactobacillus* spp. + 5 ml of sterile LAPTg medium and the given *L. monocytogenes* strain + 5 ml of sterile LAPTg medium.

The prepared mixtures were incubated at 37°C for up to 72 hours. The number of *Lactobacillus* spp. and *L. monocytogenes* in mixed cultures was assessed after 0, 24, 48 hours of incubation. 10-fold serial dilutions in PBS were made and 100 μl was plated onto Rogosa Agar (Merck) for *Lactobacillus* and OXFORD agar (Oxoid) for *L. monocytogenes*. Cultures were incubated under microaerophilic (*Lactobacillus* spp.) and aerobic (*L. monocytogenes*) conditions at 35°C for 3 days and 48 hours at 37°C, respectively. The number of colonies was expressed as CFU × ml^{-1}.

To determine the ability of *Lactobacillus* spp. to multiply (with and without *L. monocytogenes)* during the experiment, the multiplication factor (F) was calculated according to the formula:

$$F = a/b, \text{ where:}$$

F – the multiplication factor; a – the initial number of *Lactobacillus* spp. bacteria after mixtures preparation [log CFU×ml^{-1}]; b – the number of *Lactobacillus* spp. bacteria after 48-hour incubation [log CFU×ml^{-1}].

Lactobacillus spp. antagonism in aerobic conditions against *L. monocytogenes*

The lawn plates on Rogosa Agar (Merck) were made for all reference strains of *Lactobacillus* spp. and their mix. After 24 h (35°C, microaerophilic atmosphere) agar discs with the grown colonies of *Lactobacillus* spp. (LAC, LFE, LGA, LPL and LACTO MIX) were cut with the sterile cork borer.

For each of the tested *L. monocytogenes* strains, a suspension of 0.5 McF in PBS (Avantor) was prepared and spread evenly on Columbia Agar with 5% sheep blood (bioMérieux). Next, agar discs of *Lactobacillus* spp. culture was placed on such a plate. The negative control were plates with sterile agar discs. Plates were incubated 48 h (aerobic conditions) at 37°C and growth inhibition zones around the agar discs were measured [diameter in mm]. The experiment was carried out in triplicate.

Suspensions of 10 μl of *Lactobacillus* spp. (0.5 McF) in PBS (Avantor) were plated onto Rogosa Agar (Merck) and were incubated (microaerophilic conditions, 37°C, 24 h). Then chloroform (Sigma-Aldrich) soaked sterile gauze pad was placed in a closed plate with *Lactobacillus* spp. culture for 20 min to kill microbes. The gauze was then removed and the plates were left in a sterile laminar chamber to allow chloroform evaporation (30 min.). *Lactobacillus* spp. colonies were removed from the plates with a sterile cotton swab. The plates were then covered with tempered BHI (Brain Heart Infusion) Agar (bioMérieux) containing the suspension (1 McF) of *L. monocytogenes* culture (250 μl of the suspension to 12 ml of agar). The negative control was *L. monocytogenes* culture on Rogosa Agar (Merck). After incubation, the zones of inhibition of *L. monocytogenes* growth were measured and expressed in millimetres [mm].

Statistical analysis

The statistical analysis was performed using the STATISTICA 13.0 PL program (StatSoft). The significant differences of bacteria number between different experimental conditions were checked with a one-way analysis of variance and a non-parametric Bonferroni posthoc test at significance level $\alpha = 0.05$.

The significant differences of inhibition zone of *L. monocytogenes* growth between *Lactobacillus* spp. strains were calculated with a one-way analysis of variance and the Tukey posthoc test at significance level $\alpha = 0.05$. To check significant differences of inhibition zone of *L. monocytogenes* growth, depending on the *Lactobacillus* spp. and *L. monocytogenes* strain, multi-way analysis of variance and the Tukey posthoc test at significance level $\alpha = 0.05$ were applied.

Results

Assessment of *Lactobacillus spp.* number in cultures without *L. monocytogenes* and in mixed cultures with *L. monocytogenes* strains

We showed that the number of *Lactobacillus* spp. in the culture without *L. monocytogenes* and mixed culture increased together with the incubation time (Fig. 1A). The highest number of *Lactobacillus* spp. was observed in the LACTO MIX culture without *L. monocytogenes* (an increase of 9.79 log CFU × ml^{-1}, 48 h incubation). The slowest growth was demonstrated for the LGA strain with *L. monocytogenes* (increase by 5.40 log CFU × ml^{-1}, 24 h incubation). The increase of *Lactobacillus* spp. number, after 48 hours of *Lactobacillus* spp. culture with *L. monocytogenes*, ranged from 7.55 log CFU × m^{-1} (24 h) to 8.80 log CFU × ml^{-1} (48 h). The best growth in the presence of *L. monocytogenes* showed LPL whereas the slowest growth rate was found for LGA (Fig. 1A). The multiplication factor calculated for the tested *Lactobacillus* spp. strains ranged from 1.29 (LPL suspension without LMO) to 1.65 (LACTO MIX without LMO) (Fig. 1B).

Assessment of *L. monocytogenes* number in the culture with and without *Lactobacillus spp.*

The initial number of *L. monocytogenes* was 10^6 CFU × ml^{-1} and increased during incubation to 10^8–10^9 CFU × ml^{-1}, depending on the tested strain. In the experimental variant without *Lactobacillus* spp. the increase of *L. monocytogenes* number ranged from 6.67 log CFU × ml^{-1} (0h) to 9.00 log CFU × ml^{-1} (48h) (Fig. 2A). Lactobacilli had the antagonistic effect on *L. monocytogenes*. Regardless of the *Lactobacillus* spp. species and the *L. monocytogenes* strain, a statistically significant decrease in the number of *L. monocytogenes* was observed after 24 and 48 hours of cultivation (Fig. 2A). The mean of *L. monocytogenes* number at the initial time point ranged from 6.67 log CFU × ml^{-1} to 7.37 log CFU × ml^{-1} (Fig. 2A). There were no statistically significant differences in the reduction of *L. monocytogenes* number between particular, single strains of *Lactobacillus* spp. used in the co-culture. The highest antagonistic activity against *L. monocytogenes* had LACTO MIX culture (reduction number of bacteria was 4.79 log CFU × ml^{-1} after 24h incubation and 1.82 log CFU × ml^{-1} after 48 h incubation) (Fig. 2A, B).

The number of *L. monocytogenes* in such culture was statistically significantly lower compared to the number of *L. monocytogenes* incubated with a single species of *Lactobacillus* spp. (Fig. 2A). The reduction of *L. monocytogenes* number ranged from 1.99 log CFU × ml^{-1} (LAC) to 5.95 log CFU × ml^{-1} (LACTO MIX). Among the individual *Lactobacillus* strains, LPL reduced *L. monocytogenes* number most efficiently, whereas the least effective was LAC (Fig. 2B). The average number of *L. monocytogenes* after 24-hour culture with *Lactobacillus* spp. was 4.18 log CFU × ml^{-1} and 2.23 log CFU × ml^{-1} for LMO 7 and LMO 4 strains, respectively (Fig. 2C).

Lactobacillus spp. antagonism in aerobic conditions against *L. monocytogenes*

The greatest efficacy against *L. monocytogenes* was demonstrated in the mixed culture with LACTO MIX. The diameter of *L. monocytogenes* growth inhibition zone around the agar disc with the mixed culture of *Lactobacillus* spp. was 18.38 mm and was statistically significantly higher compared to the size of the zones around the discs with the individual lactobacilli strains tested (Fig. 3A).

The most effective among the single cultures of the tested *Lactobacillus* spp. species was the LPL strain

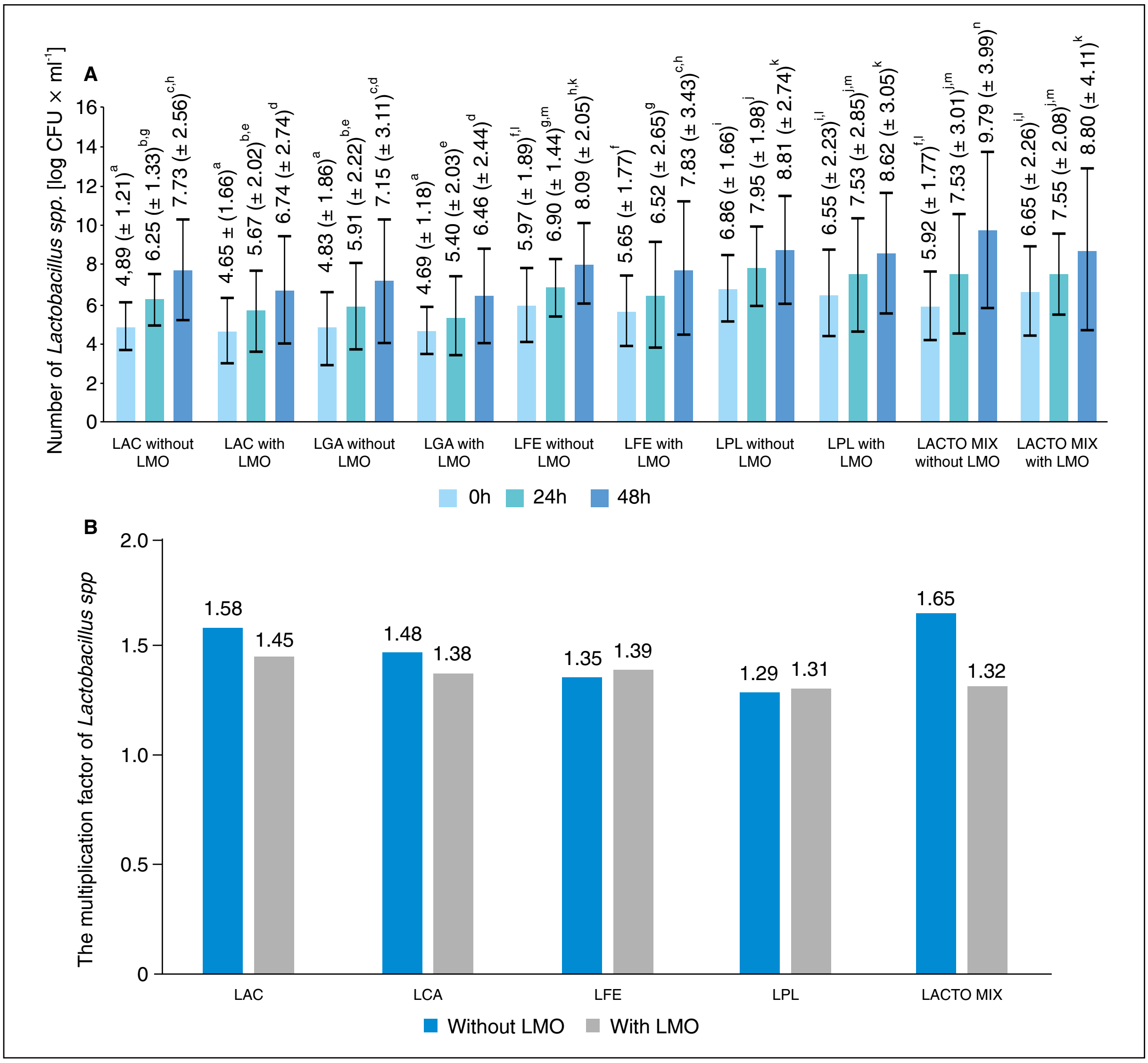

Figure 1. A. Changes in the number of *Lactobacillus* spp. in mixed culture with *L. monocytogenes.* **B.** The multiplication factor of *Lactobacillus* spp. LAC — *L. acidophilus* ATCC 314, LFE — *L. fermentum* ATCC 9338, LGA — *L. gasseri* ATCC 19992, LPL — *L. plantarum* ATCC 8014

(12.83 mm inhibition zone). The LAC strain was the least effective in controlling *L. monocytogenes* number (the average diameter of the inhibition zone of 8.50 mm). The obtained results showed that the antibacterial effectiveness of aerobic metabolites produced by *Lactobacillus* spp. depended on the tested *L. monocytogenes* strain (Fig. 3C). The most susceptible to aerobic metabolites of *Lactobacillus* spp., regardless of *Lactobacillus* species, was the LMO 4 strain (the average growth inhibition zone of 18.53 mm). The LMO 7 strain was the least sensitive to the aerobic metabolites of *Lactobacillus* spp. (the average growth inhibition zone of 6.80 mm) (Fig. 3C).

We showed that the LACTO MIX culture most effectively inhibited the growth of *L. monocytogenes*. The average diameter of the inhibition zone of the pathogenic bacteria growth on such plates was 11.67 mm and was statistically significantly bigger compared to the size of the zones on the plates with a single *Lactobacillus* spp. strain (Fig. 3B). Among individual cultures of the tested *Lactobacillus* spp. strains the highest efficacy against *L. monocytogenes* was found for LPL cultures (diameter of *L. monocytogenes* inhibition zone was 7.46 mm). The least effective in inhibiting the growth of *L. monocytogenes* was the LAC strain. The diameter of the growth inhibition zone was 4.75 mm and was

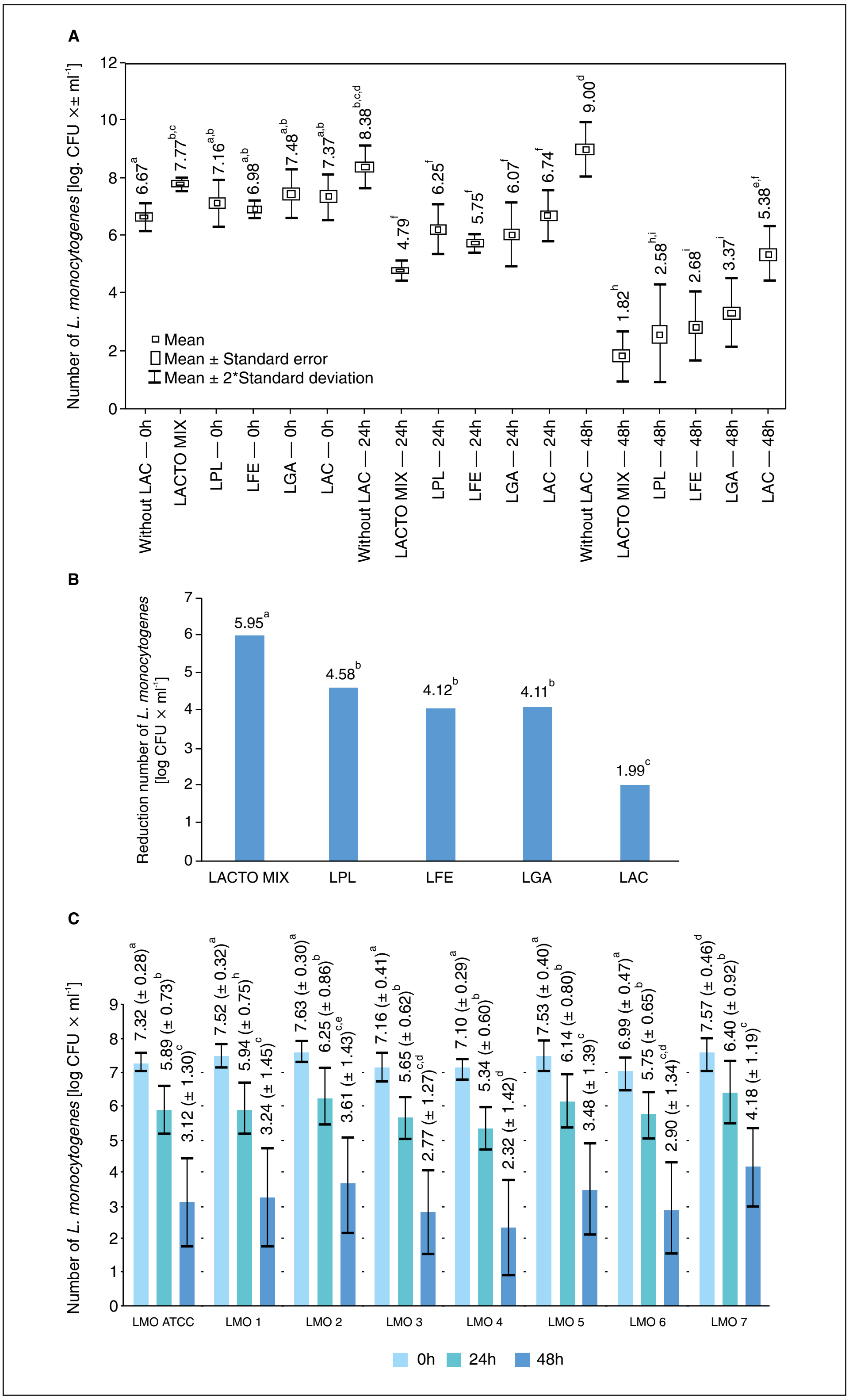

Figure 2. A. Changes in the number of *L. monocytogenes* in mixed culture with *Lactobacillus* spp. and without *Lactobacillus* spp. **B.** Decreases in *L. monocytogenes* number [log CFU × ml^{-1}] during 48 h of culture with *Lactobacillus* spp. Strains. **C.** Changes in the number of *L. monocytogenes* in the mixed culture with *Lactobacillus* spp. LAC — *L. acidophilus* ATCC 314, LFE — *L. fermentum* ATCC 9338, LGA — *L. gasseri* ATCC 19992, LPL — *L. plantarum* ATCC 8014; a,b,c,… — values marked with different letters differ statistically significant, *standard deviation, CFU — colony forming units

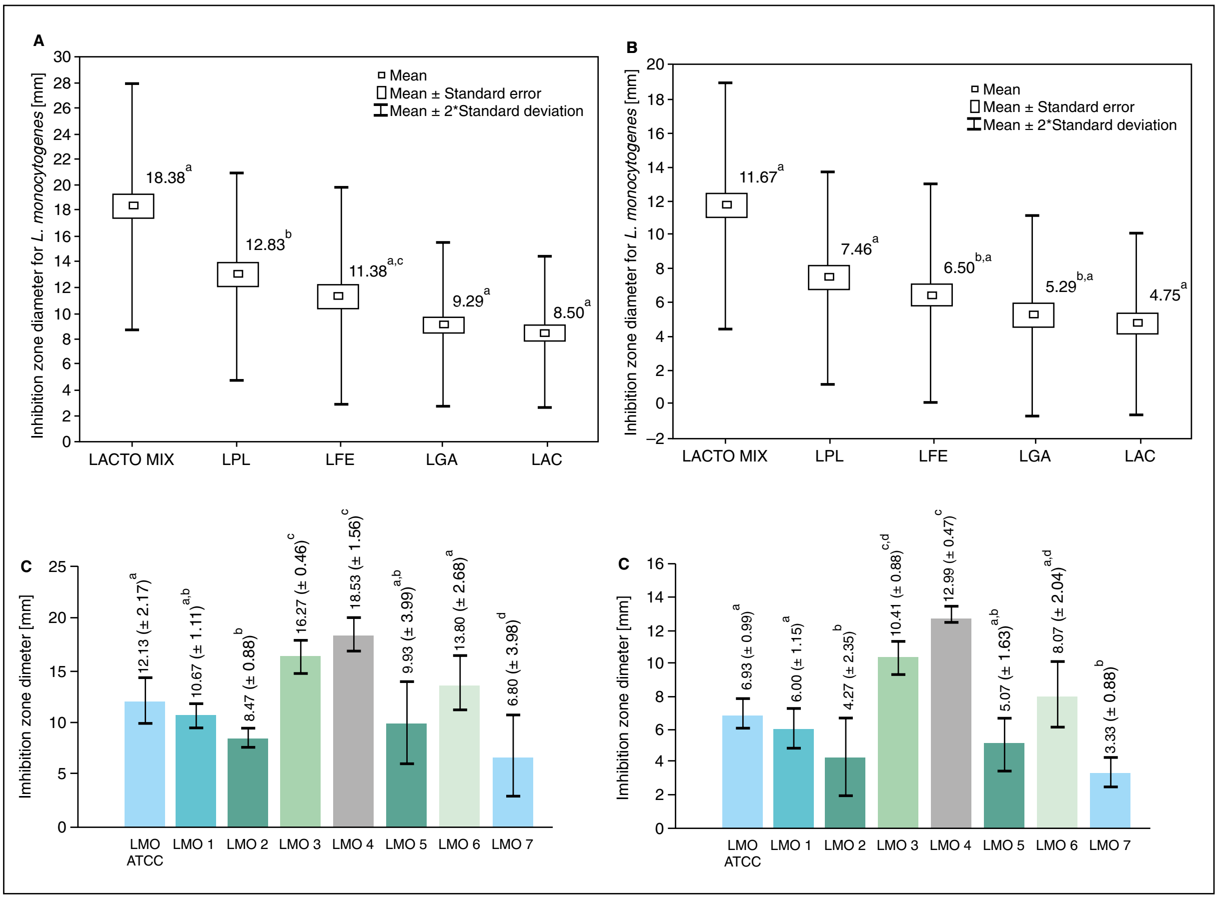

Figure 3. A. The effect of aerobic metabolites produced by *Lactobacillus* spp. strains on the size of growth inhibition zones of *L. monocytogenes*. **B.** The effect of anaerobic metabolites produced by *Lactobacillus* spp. strains on the size of growth inhibition zones of *L. monocytogenes*. **C.** The mean size of growth inhibition zones of *L. monocytogenes* due to the action of *Lactobacillus* spp. in aerobic condition. **D.** The mean size of growth inhibition zones of *L. monocytogenes* due to the action of *Lactobacillus* spp. in anaerobic conditions. LAC — *L. acidophilus* ATCC 314, LFE — *L. fermentum* ATCC 9338, LGA — *L. gasseri* ATCC 19992, LPL — *L. plantarum* ATCC 8014, a,b,c,… — values marked with different letters differ statistically significant, *standard deviation

statistically significantly smaller compared to the mixed LACTO MIX culture and the LPL culture (Fig. 3B).

It was shown that the sensitivity of *L. monocytogenes* to anaerobic metabolites of *Lactobacillus* spp. was strain-dependent (Fig. 3D). LMO4 strain was the most sensitive to bacteriocins, regardless of *Lactobacillus* species (the average inhibition zone of 12.99 mm). The LMO7 strain was the most resistant to *Lactobacillus* spp. in this variant of the experiment (the average inhibition zone of 3.33 mm) (Fig. 3D).

Discussion

The vagina of women is a natural habitat for many bacterial species, among which the predominant group are *Lactobacillus* spp. These bacteria, by secreting antimicrobial compounds, create a protective barrier against pathogenic microorganisms that cause urogenital infections [1]. One of the pathogens, dangerous especially for pregnant women, is *L. monocytogenes*. The available literature does not include studies assessing the effect of individual strains of *L. acidophilus, L. fermentum*, *L. gasseri*, *L. plantarum* and their mixture on the growth of pathogenic *L. monocytogenes*. So far, attention was paid mainly to the antagonistic properties of lactobacilli against such pathogens as *Streptococcus agalactiae*, *Gardnerella vaginalis*, and *Prevotella bivia*.

Our study showed that the mixed culture of *Lactobacillus* spp. has the highest antagonistic activity against *L. monocytogenes*. This supports the thesis that the best elimination of pathogenic microorganisms is guaranteed by the use of the culture of several *Lactobacillus* spp. strains, appropriately selected for a given female population [2, 11, 12]. Among the individual tested *Lactobacillus* spp. strains the most effective in reducing *L. monocytogenes* number was *L. plantarum*, while the smallest activity had *L. acidophilus*. Bodaszewska-Lubas et al. [13] evaluated the effect of antimicrobial properties of *L. lactis, L. plantarum* and *L. sakei* against *S. agalactiae*. They also observed that *L. plantarum* was the most effective in controlling the pathogen number, while *L. lactis* slightly inhibited the growth of *S. agalactiae*, and *L. sakei* did not exhibit antagonistic properties against the tested bacterium [13]. In turn, Atassi et al. [11] studying the effect of *L. acidophilus, L. crispatus, L. gasseri* and *L. jensenii* on the female genital tract pathogens: *G. vaginalis* and *P. bivia* found that *L. gasseri* was the most effective. In the presented study, this species displayed a moderate antagonistic activity. The effect of *Lactobacillus* spp. on other pathogens, i.e. *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, *S. agalactiae, Escherichia coli*, *L. monocytogenes, Candida* spp. was also described [2, 12, 14]. Also, Matu et al. [15] showed the inhibitory effect of *Lactobacillus* spp. on the pathogenic bacteria *P. bivia*, *G. vaginalis* and *Mobiluncus* spp. The inhibition of the growth of pathogenic microorganisms correlated with the production of organic acids such as lactic acid, hydrogen peroxide and bacteriocins by *Lactobacillus* spp strains. The growth inhibition zones were 1.5–8.0 mm for *G. vaginalis*, 1.0 - 8.0 mm for *Mobiluncus* spp. and 1.5–7.0 mm for *P. bivia*. They also demonstrated that *L. acidophilus* strain was the most effective in controlling pathogenic microorganisms [15]. In this study, the most effective among the single cultures of the tested *Lactobacillus* spp. species was the LPL strain (12.83 mm inhibition zone). Sabia et al. [16] showed that *L. fermentum* CS57 secreted a bacteriocin-like substance (BLS) with antagonistic activity against *S. agalactiae* and *Candida albicans*. In turn, Dembélé et al. [17] showed that mainly lactic acid is responsible for inhibiting the growth of pathogenic microorganisms and to a lesser extent bacteriocins secreted by *Lactobacillus* spp. strains. They also showed that the antimicrobial activity of *Lactobacillus* spp. against *L. monocytogenes* was lower compared to *S. aureus* and *Enterobacteriaceae*. The inhibition zone of *L. monocytogenes* growth ranged from 1.0 mm to 15.0 mm [17]. Gil et al. [12] showed that *L. salivarius* was the best producer of lactic acid. In turn, the study of Hütt et al. [18] found that the most efficiently lactic acid was produced by *L. gasseri*, followed by *L. crispatus* and *L. jensenii*. In the presented study, it was observed that both aerobic metabolites and bacteriocins secreted by *Lactobacillus* spp. strains inhibited *L. monocytogenes*. However, *Lactobacillus* spp. antagonism was higher under aerobic than anaerobic conditions. The sensitivity of *L. monocytogenes* in anaerobic conditions was strain-dependent. The susceptibility of tested strains to metabolites of *Lactobacillus* spp. was varied. In the available literature, no works that would unambiguously explain this phenomenon have been found. Therefore, more research is needed in this area. The resistance to antimicrobials can be inherent or acquired (e.g. in response to stress exposure). The resistance of *L. monocytogenes* strains to antibacterial metabolites of *Lactobacillus* spp. can be associated with a decreased expression of Man-PTS genes (Mannose Phosphotransferase System), responsible for the import and phosphorylation of sugars such as glucose and mannose. It has been speculated that the changes in gene expression result from the process leading to metabolic variability rather than a spontaneous mutation [19]. The role of *anrB* (encoding the permease component of an ABC transporter), *lmo222* (encoding the penicillin-binding protein) and *dltA (*responsible for the cell wall synthesis) genes in the tolerance of *L. monocytogenes* to bacteriocins has also been reported [20].

Conclusions

Lactobacillus spp. plays a key role in controlling the growth of pathogenic *L. monocytogenes* in the woman's vagina. The best results give the application of the mixed culture of a few strains. Nonetheless, there is a need for further research to accurately determine the concentration of *Lactobacillus* spp. metabolites and to understand the mechanism of their action on pathogenic bacteria, including *L. monocytogenes.*

Funding source: *This research was funded by the Nicolaus Copernicus University with funds from the maintenance of the research potential of the Department of Microbiology PDB WF 536.*

Conflict of interest: *The authors declare that they have no conflict of interest.*

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