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Synergistic Antimicrobial Effect of Raspberry (*Rubus idaeus* L., Rosaceae) Preparations and Probiotic Bacteria on Enteric Pathogens

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Due to the increasing microbial tolerance to commonly used food preservatives, as well as growing consumer awareness of their adverse impact on human health, alternative methods of pathogens reduction in food are widely investigated. The aim of this research was to examine the antimicrobial activity of red raspberry pomace and seed preparations against enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Listeria monocytogenes*, and probiotic *Lactobacillus rhamnosus* strain. The combined action of LAB (lactic acid bacteria) and raspberry preparations on the pathogenic species was also evaluated. The results of our study showed no or weak antibacterial effect of raspberry preparations on the pathogenic bacteria tested. Regardless of preparation concentration (1.0 or 2.0 mg/mL), the bacteria number after 48-h incubation was usually higher than in the culture at the initial stage and varied from 10^5 to 10^7 cfu/mL. On the other hand, probiotic *Lactobacillus rhamnosus* strain caused a significant reduction in the enteric pathogen count after 24-h co-culture with LAB. The concentrations of both *Salmonella* serotypes were below the detection limit of the analytical methods applied. Moreover, the combined use of LAB and raspberry preparations resulted in the total elimination of *Salmonella* strains and the reduction in *L. monocytogenes* number from 10^5 to 10^2–10^4 cfu/mL after 24-h co-culture. EHEC revealed the highest resistance to the mixed culture effect. The synergic antimicrobial effect suggests the possibility of applying probiotic bacteria and berry preparations as natural antimicrobial agents in the food industry.

INTRODUCTION

Microbiological contamination of food is of great concern for the food industry. A considerable number of foodborne pathogens linked to serious illness and foodborne outbreaks (*e.g. Salmonella* spp., EHEC, *Listeria monocytogenes*, *Campylobacter jejuni*) have recently gained an intense attention of epidemiologists. There are various types of preservatives used to minimize the risk related to foodborne infections. However, due to the high prevalence of antibiotic-resistant microorganisms in food, increasing microbial tolerance to conventional food preservation methods is observed. Moreover, common consumer awareness of the adverse impact of chemicals on human health prompts a growing interest in the potential use of natural preservatives.

Natural preservatives of different origins; *e.g.* animal, plant, and microbiological; have been widely studied and

their antimicrobial effectiveness remains undoubtful [Baptista *et al.*, 2020; Harich *et al.,* 2018; Salaheen *et al.,* 2016]. Their plant-derived representatives show direct antibacterial effects on the growth and metabolism of microorganisms or may indirectly modify their antibiotic resistance [Stefanovic *et al.,* 2012]. The application of essential oils and plant extracts as preservatives usually provides additional health benefits associated with the presence of specific active substances in their composition. The group of particular importance are phenolics, revealing high antibacterial and antifungal properties [Negi, 2012; Pandey *et al.,* 2017].

Fruits of red raspberry (*Rubus ideaus* L.), one of the most popular berry crops in the world, are commonly consumed both as fresh and as ingredients in processed food products. They are a rich source of various bioactive phenolic compounds, among which anthocyanins, ellagitannins, and conjugates of ellagic acid and quercetin are of great significance [Burton-Freeman *et al.,* 2016; Klewicka *et al.,* 2016; Stój *et al.,* 2006]. The total phenolic content of red raspberries varies from 200 to 500 mg/100 g of fresh matter; however,

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the content of anthocyanins varies between cultivars [Grume-zescu, 2016; Simmonds & Preedy, 2015]. Among the antho-cyanins found in red raspberries, cyanidin 3-*O*-sophoroside, cyanidin 3-*O*-glucoside, and pelargonidin 3-*O*-sophoroside are the most common and account for 79.8%, 14.2%, and 6.0% of the total anthocyanin content, respectively [Coultate, 2016; Shahidi & Alasalvar, 2016]. Sanguiin H-6 and lam-bertianin C are the major raspberry ellagitannins; however, their content and profile are genetically-determined and vary among different raspberry cultivars [Klewicka *et al.,* 2016; Vrhovsek *et al.,* 2009].

Fresh raspberries are the best source of bioactive com-pounds. Due to the short harvesting period, fresh raspberry fruits are mostly frozen or processed into concentrate, pre-serves, canned products, aseptic packs, and juice. During in-dustrial juice production, a significant amount of the biologi-cally-active ingredients remains in the fruit pomace and seeds [Schieber *et al.,* 2001]. These processed products still contain valuable substances and, therefore, increase the bio-potential of the by-products [Balasundram *et al.,* 2006]. The profiles of their phenolic compounds are similar to fresh fruits but the phenolic content of seeds and pomace is lower than that of fruit. Although anthocyanins in raspberries are quantita-tively the most important polyphenols, they are accumulated mostly in the flesh and transferred to juice in the process of its production, which is why they are found in little quantities in the seeds and pomace. Contrary to the anthocyanins, ella-gitannins accumulate mainly in the pomace. It should be em-phasized that 80% of the dried raspberry pomace consists of the seeds. The fine grinding of the native pomace results in the damage to the seed coat, increasing the availability of the ac-cumulated valuable seed compounds [Fotschki *et al.,* 2017].

Besides their beneficial effect on human health [Jimenez--Garcia *et al.,* 2013; Paredes-López *et al.,* 2010], phytochemi-cals in red raspberries show antimicrobial activity against many human pathogens [Nile &Park, 2014]. The complex phenolic polymers, such as ellagitannins, are particularly ac-tive in this respect [Heinonen, 2007; Małecka *et al.,* 2003]. The results of many research suggest various mechanisms related to the pathogen growth inhibition by berry phenolics, *e.g.* disruption of cytoplasmic membrane structure, permea-bilization of the cell membrane, inhibition of extracellular enzyme secretion, or direct effect on microbial metabolism processes [Puupponen-Pimiä *et al.,* 2005a]. Since the colo-nization of intestinal epithelium is crucial for the food-borne bacterial infections, the anti-adherence activity of active sub-stances in berries is also linked to their antimicrobial effect [Das *et al.,* 2017; Puupponen-Pimiä *et al.,* 2005b].

Among the natural substances used as natural food pre-servatives, lactic acid bacteria (LAB) metabolites demonstrate great antimicrobial potential [Adetoye *et al.,* 2018]. Organic acids, hydrogen peroxide, and bacteriocins inhibit the growth of intestinal and food-borne pathogens [Chen *et al.,* 2019].

Due to the antibacterial features of both, LAB and rasp-berry active substances (phenolic compounds), the putative synergic action of their combination, increasing the beneficial effect on human health, can be presumed. In this context, the co-application of these biocomponents in functional food (fer-mented milk products, juices) is an issue of a special interest.

The aim of this study was to investigate the antibacterial effects of dried preparations obtained from raspberry pom-ace, seed, and seedless pomace after industrial juice produc-tion against enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Listeria monocytogenes*, and probiotic *Lactobacillus rhamnosus* strain. The synergistic effect of LAB and raspberry preparations on the pathogenic species was also examined.

MATERIALS AND METHODS

Raspberry preparations

Native raspberry (*R. ideaus*) pomace (preparation P), seed preparation (preparation S; > 95% seed content), and rasp-berry seedless pomace (preparation W; <5% seed content) were supplied by Agro-Bio-Produkt Sp. z o.o. (Grodkowice, Poland). The native P pomace was a biomass residue after in-dustrial juice production and drying in the SB-1.5 rotary drum dryer (AGROMECH Co., Rogozno Wlkp., Poland). The seed preparation was a commercial product intended for high-qual-ity oil production. It was obtained by the producer with the aid of two layers of a mesh screen taking into account the average dimension (length, width, and thickness) of *R. ideaus* seeds. The W preparation was a residue obtained after the S prepara-tion production. In order to standardize all three preparations, their samples were ground in a cryogenic environment (Freezer Mill 6870 SPEX, SamplePrep. Inc., Stanmore, UK) to obtain particles smaller than 0.65 mm. That process made it possible to get through the seed coat and to preserve the bioactive com-ponents on the preparations.

Proximate composition of the preparations

The official methods of the Association of Official Analyt-ical Chemists [AOAC, 2007] method were used to determine the proximate analysis of the raspberry preparations: protein content, 920.152; dry matter and ash content, 940.26; ether extract, 930.09; total dietary fiber (TDF) content, 985.29, and insoluble dietary fiber (IDF) content, 991.42. The soluble dietary fiber (SDF) content was estimated as the difference between TDF and IDF content. All analyses were performed in triplicate.

Phenolics content of the preparations

A three-step extraction procedure with acetone-water--formic acid (70:29.9:0.1, *v/v/v*) was applied to extract pheno-lics from preparations tested. First, 500 mg of ground materi-al was vortexed with 4 mL of the solvent. After sonication for 15 min and centrifugation at 4,800 × g, the extract obtained was transferred into a flask. The vortexing was repeated twice with 3 mL of the solvent.

The content of ellagitannins was determined using a Smartline chromatograph (Knauer, Berlin, Germany) with a degasser (Manager 5000), two pumps (P1000), autosampler (3950), thermostat, and photodiode array detector (2800). Ellagitannins were separated on a Gemini C18 column: 250 × 4.6 mm, 5 μm, 110 Å (Phenomenex, Torrance, CA) by gradient elution with solvent A (0.05% [*v/v*] phosphoric acid--water) and solvent B (63:20:17 [*v/v/v*] acetonitrile-methanol-

-water with 0.05% phosphoric acid). The column temperature was set at 35°C, the flow rate was 1.25 mL/min, and the gradient program was as follows: 0–5 min, 5% (*v/v*) B; 5–30 min, 5–28% (*v/v*) B; 30–40 min, 28–73% (*v/v*) B; 40–45 min, 73% (*v/v*) B; 45–47 min, 73–5% (*v/v*) B; and 47–56 min, 5% (*v/v*) B. The injection volume was 20 μL.

ClarityChrom v. 3.0.5.505 software (Knauer, Berlin, Germany) was applied for data collection. The standards applied were ellagic acid, sanguiin H-6, lambertianin C, and bis-HHDP-glucose (Extrasynthese, Genay, France).

The excess phloroglucinol degradation method was applied to determine the content of procyanidins. A methanol solution (0.8 mL) with phloroglucinol (75 g/L) and ascorbic acid (15 g/L) were added to a 20 mg sample. To start the reaction, 0.4 mL of 0.2 M hydrochloric acid in methanol were added. The reaction proceeded at 50°C for 30 min and was stopped by adding 0.6 mL of a 40 mM sodium acetate solution in an ice bath. The samples were centrifuged at $3,600 \times g$ for 5 min, diluted with a 40 mM sodium acetate solution, and analyzed using a Smartline chromatograph with a P2800 UV-Vis detector (both from Knauer, Berlin, Germany), an RF-10AXL fluorescence detector (FD) (Shimadzu, Tokyo, Japan), and a Gemini C18 column (110 Å, 5 μm, 250 $\times$ 4.60 mm) (Phenomenex, Torrance, USA). Phase A consisted of acetic acid and water (2.5:97.5, *v/v*), while phase B of acetonitrile and water (80:20, *v/v*). The applied gradient, with the flow rate of 1 mL/min at 25°C, was as follows: 0–10 min, 4–7% (*v/v*) B; 10–27 min, 7–30% (*v/v*) B; 27–29 min, 30–70% (*v/v*) B; 29–34 min, 70% (*v/v*) B; 34–35 min, 7–40% (*v/v*) B; and 35–40 min, 4% (*v/v*) B. Comparison of the retention times and UV-Vis spectra with those of standards: (−)-epicatechin, (+)-catechin, (−)-epicatechin−phloroglucinol adduct, and (+)-catechin−phloroglucinol adduct, was used for identification. The excitation and the emission wavelengths were 278 nm and 360 nm, respectively.

Microorganisms tested

The bacterial strains: *Salmonella* Typhimurium, *Salmonella* Enteritidis, EHEC, and *Listeria monocytogenes* from the collection of the Department of Microbiology and Food Technology, UTP University of Science and Technology in Bydgoszcz, Poland, were used in the study. *Lactobacillus rhamnosus* strain was obtained from a commercial probiotic preparation.

Antimicrobial activity of raspberry preparations

The liquid culture method was used to study the effect of P, W, and S raspberry preparations on the bacteria tested. The liquid growth medium LAPTg (10 mL) was inoculated with 100 μL of a bacterial overnight culture. The initial bacterial culture titer was 10^8 cfu/mL. Raspberry preparations were suspended in 10 mL of inoculated media to a final concentration of 1.0 or 2.0 mg/mL. Preparations doses were chosen according to Nohynek *et al.* [2006] and Puupponen-Pimiä *et al.* [2001] methodologies. The cultures were incubated at 37°C for 48 h. Bacterial culture samples were taken four times during an incubation time – immediately after inoculation (0) and after 6, 24, and 48 h. The samples were diluted by introducing 1 mL of the culture into test tubes containing sterile 0.9%

NaCl solution (10^1–10^9). Next, 0.1 mL of each dilution was transferred and distributed on the medium surface, using the spread plate technique. The following selective growth media were used for bacteria isolation: EHEC – Endo Agar (Merck, 1.04044), *Salmonella* strains – BPL Agar (Merck, 1.10747), *L. monocytogenes* – ALOA (Merck, 1.00427), and *L. rhamnosus* – Rogosa Agar (Merck, 1.05413). The incubation of the tested microorganisms was conducted at 37°C for 24–48 h. Bacterial cultures with no raspberry preparations were used as controls. Each experiment was performed in triplicate.

Antimicrobial activity of probiotic *Lactobacillus* strain

The co-culture (mixed cultures, associated cultures) method was applied to estimate the interaction between *L. rhamnosus* and pathogenic strains. Co-cultures were obtained by adding 100 μL of a 24-h *L. rhamnosus* pure culture and 100 μL of an individual pathogen pure culture to 10 mL of the liquid growth medium LAPTg. The initial titer of both bacterial cultures was 10^8 cfu/mL. To examine the effect of LAB on the pathogens tested, the co-cultures were incubated at 37°C for 24 h. Culture samples were taken five times during the incubation time – immediately after inoculation (0) and after 15, 18, 21, and 24 h. The samples were diluted by introducing 1 mL of the culture into test tubes containing sterile 0.9% NaCl solution (10^1–10^9). Then, 0.1 mL of each dilution was plated out by spread plating on different selective solid media, allowing the growth of only one of the two microorganisms composing the mix. The cultures were incubated at 37°C for 24–48 h. Pure cultures of each bacteria were used as controls. The selective media and incubation conditions were similar to those described above. Each experiment was performed in triplicate.

Evaluation of the synergistic effect of raspberry preparations and *Lactobacillus* on the pathogens tested

Co-cultures of individual pathogenic bacterial strains and *L. rhamnosus*, enriched with raspberry preparations, were used to assess the combined action of LAB and raspberry compounds. The liquid growth medium LAPTg (10 mL) was inoculated with 100 μL of the *L. rhamnosus* overnight culture and 100 μL of the pathogen overnight culture. The initial titer of both bacterial cultures was 10^8 cfu/mL. Then, raspberry preparations were added to the inoculated media to a final concentration of 2.0 mg/mL. The co-cultures were incubated at 37°C for 24 h. Culture samples were taken five times during the incubation time – immediately after inoculation (0) and after 15, 18, 21, and 24 h. The samples were diluted by introducing 1 mL of the culture into test tubes containing sterile 0.9% NaCl solution (10^1–10^9). Then, 0.1 mL of each dilution was plated out by spread plating on the growth media as described above. The tested microorganisms were incubated at 37°C for 24–48 h. Pure cultures of each bacteria were used as controls. Each experiment was performed in triplicate.

Statistical analysis

Statistical analysis was performed using Statistica software (*TIBCO Software* Inc., Palo Alto, CA, USA). The results are presented as mean ± standard deviation (SD). Differences between control and experimental variants were analyzed by one-way ANOVA followed by the Dunnett's test ($p < 0.05$).

RESULTS AND DISCUSSION

The chemical composition of raspberry preparations is summarized in Table 1. The content of total phenolics in raspberry preparations varied from 1665 mg/100 g in seed preparation (S) to 4253 mg/100 g in seedless pomace (W). The highest content of ellagitannins (4020 mg/100 g) was also noted in the seedless pomace preparation (W) (Table 1). Klimczak *et al*. [2011] demonstrated that most of the phenolics accumulate in the seedless fraction of the pomace; consequently, an increased concentration of these bioactive compounds was observed after the removal of seeds from the raspberry pulp.

All of the three preparations tested (W, S, P) showed no or low antimicrobial effect on the microorganisms tested, and bacterial species did not affect the raspberry inhibitory efficiency (Table 2). According to Nohynek *et al.* [2006], different bacterial species and strains demonstrate variable sensitivity to phenolic berry extracts. The results of their research showed a high efficiency of raspberry against *Helicobacter pylori* and *Bacillus cereus*. Growth inhibition of *Campylobacter jejuni* and *Candida albicans* was linked to the high content of ellagitannins in the composition of the tested extracts [Nohynek *et al.,* 2006]. In turn, Puupponen-Pimiä *et al.* [2001, 2005a] reported a low antimicrobial activity of raspberry against Gram-positive bacteria, including *L. monocytogenes.* On the other hand, raspberry extracts were strong inhibitors of Gram-negative intestinal bacteria, which might be the effect of different cell membrane structure. These findings were not confirmed by Velićanski *et al.* [2012]*,* who reported a higher, compared to G-positive, resistance of G-negative bacteria, especially with *Escherichia coli* and *Salmonella* Typhimurium to raspberry pomace extracts in agar diffusion tests. Among all G-positive bacteria tested, *L. monocytogenes* showed the lowest sensitivity. Similarly, the study of Krstic *et al.* [2014] demonstrated the antibacterial activity of the raspberry ethanol extract and juice on Gram-positive bacteria. Rauha *et al.* [2000] observed that the inhibitory action of raspberry phenolics on bacteria tested was not limited to G-positive and G-negative species only. The raspberry active compounds inhibited the growth of *Bacillus subtilis* and *Micrococcus luteus* but had only limited effect on *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis.* The results of a study by Četojević-Simin *et al.* [2015] showed that Gram-positive and Gram-negative bacteria were similarly susceptible to raspberry pomace extracts, and that the growth inhibition of various bacterial species was neither strain- nor species-dependent.

Various internal and external factors might have caused the low antibacterial activity of the tested raspberry products in our study. The berry cultivar, as well as the procedure applied to obtain the plant preparations, influence the amount and composition of phenolic compounds and the antimicrobial action of a final product [Krisch *et al.,* 2008; Krstic *et al.,* 2014; Lima *et al.,* 2019]. The antibacterial efficiency of berry preparations is also due to their concentration applied in the experimental procedure. The doses used in our study were 1.0 or 2.0 mg/mL. Nohynek *et al.* [2006] observed 0.5 to 1.5 log reduction of *C. jejuni* number after 5-h incubation with lyophilized raspberry extracts in a 1.0 mg/mL concentration. A similar dose of raspberry extracts totally inhibited the growth of

TABLE 1. Chemical composition of raspberry pomace and seed preparations.

Content	Native pomace (P)	Seedless pomace (W)	Seeds (S)
Dry matter (g/100 g)	95.4±0.0	95.3±0.0	96.5±0.2
Ash (g/100 g)	2.68±0.25	5.12±0.02	1.71±0.06
Protein (g/100 g)	11.4±0.0	18.9±0.1	10.5±0. 5
Ether extract (g/100 g)	12.2±0.1	6.00±0.01	14.1±0.2
TDF (g/100 g), including:	61.3±0.7	54.3±0.5	63.9±0.4
IDF (g/100 g)	59.2±0.7	51.4±0.5	62.7±0.4
SDF (g/100 g)	2.12±0.06	2.90±0.07	1.23±0.02
Total phenolics (mg/100 g), including:	2359±30	4253±23	1665±44
Ellagitannins	1949±18	4020±12	1211±34
bis-HHDP-glucoseisomer 1	36.5±4.0	32.7±2.2	34.6±3.2
bis-HHDP-glucoseisomer 2	41.8±2.1	3.40±0.30	38.9±2.4
Sanguiin H10 isomer 1	34.0±0.1	43.8±0.6	31.8±0.5
Lambertianin C without ellagic acid	44.9±0.9	84.8±0.5	29.1±1.4
SanguiinH10 isomer 2	0.0±0.0	65.0±1.0	31.1±1.0
Lambertianin C isomer 1	17.0±0.8	25.9±1.4	21.5±0.7
Lambertianin C isomer 2	56.4±0.0	75.7±1.0	41.9±0.0
Lambertianin C isomer 3	53.5±4.0	142±6	14.4±0.8
Lambertianin D	92.3±1.5	226±3	113±3
Lambertianin C	820±8	1998±0	375±7
Sanguiin-H6	752±5	1322±1	478±15
Ellagic acid	73.4±1.0	139±1	106±5
Procyanidins	397±12	229±11	439±9
Free catechins	13.1±0.1	3.40±0.04	14.9±0.5

TDF – total dietary fiber; IDF – insoluble dietary fiber; SDF – soluble dietary fiber.

S. Typhimurium and *E. coli* CM 871 and decreased the number of *E. coli* strain 50 by 2 logs, compared to the control culture [Puupponen-Pimiä *et al.,* 2001]. Puupponen-Pimiä *et al.* [2005a] noted a strong inhibitory effect of raspberry extracts at a low concentration (1.0 mg/mL) at the initial phase of *Salmonella* strains cultivation, followed, however, by the increase in bacteria number after 12 and 24 h of incubation. Application of the 5.0 mg/mL dose resulted in the complete elimination of *S.* Typhimurium after 2-h incubation. In our study, the effect of raspberry preparations on pathogenic bacteria was dose-independent. The number of the microorganisms tested in the initial culture varied from 10^4 to 10^7 cfu/mL and, in the majority of experimental variants, increased after 6 and 24 h of incubation. During the next 24 h, a slight reduction was observed in bacteria culture density; however, the cell count was usually higher than in the culture at the initial stage (Table 2).

TABLE 2. Effect of raspberry preparations on the bacteria count (cfu/mL).

Microorganism	Preparation dose and type		Incubation time (h)			
			0	6	24	48
EHEC	1 mg/mL	W	$3.20\times10^6\pm1.44\times10^6$	$4.00\times10^8\pm2.65\times10^7$	$2.80\times10^8\pm7.55\times10^7$	$7.67\times10^7\pm1.53\times10^{7*}$
		S	$3.00\times10^6\pm7.00\times10^5$	$3.47\times10^8\pm1.26\times10^8$	$4.43\times10^8\pm9.81\times10^7$	$5.53\times10^7\pm3.00\times10^7$
		P	$3.73\times10^6\pm9.29\times10^5$	$3.43\times10^8\pm5.77\times10^7$	$3.30\times10^8\pm1.15\times10^8$	$4.13\times10^7\pm1.69\times10^7$
	2 mg/mL	W	$4.00\times10^6\pm7.00\times10^{5*}$	$3.37\times10^8\pm2.08\times10^7$	$1.80\times10^8\pm7.21\times10^7$	$1.18\times10^8\pm3.05\times10^{6*}$
		S	$4.83\times10^6\pm1.25\times10^{6*}$	$5.13\times10^8\pm2.27\times10^8$	$2.63\times10^8\pm2.89\times10^7$	$9.03\times10^7\pm2.87\times10^{7*}$
		P	$6.40\times10^6\pm4.58\times10^{5*}$	$2.63\times10^8\pm5.77\times10^6$	$2.13\times10^8\pm7.64\times10^7$	$7.50\times10^7\pm1.51\times10^{7*}$
	Control		$1.90\times10^6\pm1.00\times10^5$	nd	nd	$1.50\times10^7\pm1.30\times10^7$
Listeria monocytogenes	1 mg/mL	W	$7.53\times10^5\pm1.74\times10^{5*}$	$6.83\times10^6\pm1.07\times10^6$	$7.93\times10^5\pm2.00\times10^5$	$4.43\times10^7\pm6.03\times10^{6*}$
		S	$7.27\times10^5\pm3.05\times10^{4*}$	$2.60\times10^5\pm3.46\times10^4$	$8.83\times10^5\pm2.75\times10^5$	$5.43\times10^7\pm2.08\times10^{6*}$
		P	$6.67\times10^5\pm5.77\times10^4$	$4.10\times10^5\pm1.73\times10^4$	$3.10\times10^6\pm1.23\times10^6$	$1.00\times10^6\pm3.61\times10^{5*}$
	2 mg/mL	W	$5.03\times10^5\pm9.50\times10^4$	$8.60\times10^6\pm3.49\times10^6$	$1.12\times10^8\pm1.50\times10^7$	$1.10\times10^7\pm2.65\times10^6$
		S	$5.20\times10^5\pm3.47\times10^4$	$7.53\times10^6\pm7.23\times10^5$	$1.68\times10^8\pm6.43\times10^6$	$2.57\times10^5\pm9.29\times10^{4*}$
		P	$5.53\times10^5\pm4.51\times10^4$	$6.40\times10^6\pm9.64\times10^5$	$1.36\times10^8\pm4.04\times10^6$	$3.17\times10^7\pm1.22\times10^{7*}$
	Control		$4.36\times10^5\pm1.21\times10^5$	nd	nd	$7.83\times10^6\pm3.01\times10^6$
Salmonella Enteritidis	1 mg/mL	W	$5.95\times10^6\pm1.40\times10^6$	$9.30\times10^8\pm2.33\times10^8$	$9.80\times10^8\pm2.44\times10^8$	$1.10\times10^7\pm7.60\times10^6$
		S	$5.90\times10^6\pm6.00\times10^5$	$8.37\times10^8\pm1.10\times10^8$	$6.83\times10^8\pm5.23\times10^8$	$2.17\times10^6\pm5.69\times10^{5*}$
		P	$6.02\times10^6\pm2.40\times10^6$	$7.17\times10^8\pm1.95\times10^8$	$1.33\times10^9\pm5.20\times10^7$	$9.83\times10^6\pm8.54\times10^6$
	2 mg/mL	W	$4.30\times10^6\pm1.04\times10^6$	$5.10\times10^8\pm7.94\times10^7$	$7.87\times10^8\pm6.35\times10^7$	$3.56\times10^7\pm2.70\times10^7$
		S	$4.00\times10^6\pm6.08\times10^5$	$4.47\times10^8\pm4.04\times10^7$	$8.43\times10^8\pm1.11\times10^8$	$1.61\times10^7\pm9.00\times10^6$
		P	$4.87\times10^6\pm1.08\times10^6$	$8.90\times10^8\pm2.01\times10^8$	$7.23\times10^8\pm5.51\times10^7$	$2.57\times10^6\pm2.62\times10^{6*}$
	Control		$7.53\times10^6\pm3.02\times10^6$	nd	nd	$4.00\times10^7\pm2.40\times10^7$
Salmonella Typhimurium	1 mg/mL	W	$4.70\times10^6\pm7.94\times10^5$	$4.50\times10^8\pm2.21\times10^8$	$9.90\times10^8\pm2.65\times10^7$	$3.80\times10^8\pm2.65\times10^7$
		S	$3.90\times10^6\pm1.31\times10^6$	$6.60\times10^8\pm2.13\times10^8$	$1.35\times10^9\pm3.23\times10^8$	$3.20\times10^8\pm2.00\times10^7$
		P	$4.57\times10^6\pm1.82\times10^6$	$5.27\times10^8\pm1.03\times10^8$	$7.57\times10^8\pm1.60\times10^8$	$2.27\times10^8\pm6.43\times10^{7*}$
	2 mg/mL	W	$6.30\times10^6\pm6.08\times10^5$	$3.00\times10^8\pm5.03\times10^7$	$8.00\times10^8\pm1.30\times10^8$	$1.01\times10^8\pm9.54\times10^{6*}$
		S	$5.50\times10^6\pm6.08\times10^5$	$3.23\times10^8\pm4.04\times10^7$	$8.03\times10^8\pm8.74\times10^7$	$3.63\times10^8\pm1.14\times10^8$
		P	$6.50\times10^6\pm1.80\times10^6$	$3.40\times10^8\pm5.20\times10^7$	$6.33\times10^8\pm3.51\times10^7$	$2.93\times10^8\pm2.09\times10^7$
	Control		$6.90\times10^6\pm1.10\times10^6$	nd	nd	$3.80\times10^8\pm7.21\times10^7$
Lactobacillus rhamnosus	1 mg/mL	W	$1.10\times10^7\pm2.00\times10^6$	$1.27\times10^8\pm6.93\times10^7$	$6.85\times10^8\pm5.07\times10^7$	$7.33\times10^7\pm2.52\times10^7$
		S	$9.00\times10^6\pm2.65\times10^6$	$9.20\times10^7\pm3.70\times10^7$	$6.99\times10^8\pm5.33\times10^7$	$8.33\times10^7\pm1.53\times10^7$
		P	$1.13\times10^7\pm3.21\times10^6$	$6.37\times10^7\pm3.51\times10^7$	$6.60\times10^8\pm4.00\times10^7$	$1.96\times10^8\pm4.51\times10^7$
	2 mg/mL	W	$1.27\times10^7\pm8.50\times10^{5*}$	$1.20\times10^8\pm9.50\times10^6$	$7.23\times10^8\pm1.63\times10^8$	$4.00\times10^7\pm2.00\times10^{6*}$
		S	$1.42\times10^7\pm2.33\times10^{6*}$	$1.50\times10^8\pm3.55\times10^7$	$8.83\times10^8\pm5.51\times10^7$	$3.87\times10^7\pm6.03\times10^{6*}$
		P	$1.57\times10^7\pm2.16\times10^6$	$1.23\times10^8\pm1.57\times10^7$	$1.01\times10^9\pm7.00\times10^7$	$3.97\times10^7\pm1.16\times10^{7*}$
	Control		$1.73\times10^7\pm1.53\times10^7$	nd	nd	$2.17\times10^8\pm7.64\times10^7$

EHEC – Enterohemorrhagic *E. coli*; W – raspberry seedless pomace preparation; S – seed preparation; P – native raspberry pomace preparation; nd – not determined; *significant difference ($p<0.05$) compared to control.

TABLE 3. Count of the pathogenic bacteria in the co-culture with *L. rhamnosus* (cfu/mL).

Microorganism	Culture conditions	Incubation time (h)				
		0	15	18	21	24
EHEC	Co-culture	$4.25 \times 10^6 \pm 3.51 \times 10^5$	$2.40 \times 10^8 \pm 4.65 \times 10^7$	$2.18 \times 10^8 \pm 7.21 \times 10^6$	$2.60 \times 10^8 \pm 3.46 \times 10^7$	$1.73 \times 10^8 \pm 1.89 \times 10^{7*}$
	Control	$4.00 \times 10^6 \pm 2.58 \times 10^6$	nd	nd	nd	$3.00 \times 10^8 \pm 4.00 \times 10^7$
Listeria monocytogenes	Co-culture	$7.00 \times 10^5 \pm 2.00 \times 10^5$	$1.27 \times 10^7 \pm 3.05 \times 10^6$	$7.27 \times 10^6 \pm 1.11 \times 10^6$	$6.20 \times 10^5 \pm 1.01 \times 10^5$	$1.33 \times 10^3 \pm 2.89 \times 10^{2*}$
	Control	$5.67 \times 10^5 \pm 1.15 \times 10^5$	nd	nd	nd	$4.03 \times 10^8 \pm 4.17 \times 10^7$
Salmonella Enteritidis	Co-culture	$4.00 \times 10^6 \pm 5.29 \times 10^5$	$2.33 \times 10^7 \pm 1.53 \times 10^6$	$1.53 \times 10^5 \pm 5.77 \times 10^3$	$9.77 \times 10^3 \pm 3.21 \times 10^2$	nf*
	Control	$3,63 \times 10^6 \pm 3.51 \times 10^5$	nd	nd	nd	$3.77 \times 10^8 \pm 3.79 \times 10^7$
Salmonella Typhimurium	Co-culture	$7.47 \times 10^6 \pm 1.07 \times 10^6$	$3.10 \times 10^8 \pm 4.00 \times 10^7$	$4.53 \times 10^7 \pm 1.53 \times 10^6$	$3.70 \times 10^4 \pm 8.88 \times 10^3$	nf*
	Control	$6.80 \times 10^6 \pm 1.06 \times 10^6$	nd	nd	nd	$6.37 \times 10^8 \pm 6.03 \times 10^7$

EHEC – Enterohemorrhagic *E. coli*; nd – not determined; nf – not found; *significant difference (p<0.05) compared to control.

Even though growth inhibition of pathogenic bacteria is a desirable result of the applied raspberry preparations, an opposite effect is expected towards probiotic LAB strains. Nohynek *et al.* [2006] observed no antagonistic action of raspberry extracts on probiotic *L. rhamnosus* strains. In the study by Puupponen-Pimiä *et al.* [2001], *Lactobacillus* strains in the liquid culture were not affected by raspberry extracts at low concentrations (1 ± 1 mg/mL). However, the growth of these bacteria was inhibited, when a five times higher concentration of raspberry extracts was used. The present research showed no antimicrobial effect of the 1 mg/mL raspberry preparation dose on *L. rhamnosus* growth in the liquid culture. On the other hand, bacteria counts were significantly lower for all combinations of raspberry preparations (concentration 2 mg/mL) with *L. rhamnosus* compared to the control (Table 2).

The co-culture of probiotic lactobacilli and enteric pathogens is a laboratory method widely used to assess relationships between microorganisms tested. Chen *et al.* [2019] reported a significant growth inhibition of *E. coli* after 48-h co-culture with lactobacilli. A probiotic combination of *L. acidophilus* and *L. rhamnosus* caused a complete elimination of *E. coli* after 12 h [Bertuccini *et al.*, 2017]. The reduction in *Salmonella* Enterica number (from 8 $\log_{10}$ to no viable) occurred between 8 and 16 h of co-culture with the two LAB strains [Adetoye *et al.*, 2018]. Our research showed a high inhibitory effect of the probiotic *Lactobacillus* against *Salmonella* strains, resulting in the reduction of pathogen count below the inoculum level within 24 h (Table 3). After 24-h co-incubation, *L. monocytogenes* number decreased from the initial 7.00×10^5 cfu/mL to 1.33×10^3 cfu/mL, while in the control pure culture it exceeded 10^8 cfu/mL (Table 3). Although the concentration of enterohemorrhagic *E. coli* cells in the co-culture and control culture remained at the same level of 10^8 cfu/mL during the final analysis, a significant inhibitory activity of *L. rhamnosus* against EHEC was reported (Table 3).

Although various mechanisms, including the production of bacteriocins and H_2O_2, are linked to pathogen growth inhibition by LAB probiotic strains, the acidification of the growth medium is suggested to be the crucial factor responsible for the co-culture reduction efficiency [Chen *et al.*, 2019; Delley *et al.*, 2015]. On the other hand, Shen *et al.* [2014] reported a weak effect of the medium pH on the antibacterial activity of blueberry ethanol extract. A weak correlation between berry juices acidity and their antimicrobial effect was also noted by Krisch *et al.* [2008].

TABLE 4. Count of *Lactobacillus rhamnosus* (cuf/mL) in the co-culture with the pathogenic bacteria and raspberry preparations in the concentration of 2.0 mg/mL.

Co-culture conditions		Incubation time (h)	
		0	24
EHEC		$1.93 \times 10^7 \pm 3.51 \times 10^{6*}$	$6.27 \times 10^8 \pm 1.10 \times 10^7$
EHEC + raspberry preparations	W	$4.90 \times 10^6 \pm 1.25 \times 10^{6*}$	$1.37 \times 10^9 \pm 1.81 \times 10^{8*}$
	S	$6.63 \times 10^6 \pm 2.03 \times 10^6$	$1.45 \times 10^9 \pm 1.12 \times 10^{8*}$
	P	$5.83 \times 10^6 \pm 2.06 \times 10^{6*}$	$1.14 \times 10^9 \pm 1.11 \times 10^{8*}$
Listeria monocytogenes		$1.00 \times 10^7 \pm 1.70 \times 10^6$	$6.23 \times 10^8 \pm 4.15 \times 10^7$
Listeria monocytogenes + raspberry preparations	W	$4.53 \times 10^6 \pm 2.54 \times 10^{6*}$	$8.77 \times 10^8 \pm 1.00 \times 10^{8*}$
	S	$9.07 \times 10^6 \pm 2.57 \times 10^6$	$8.33 \times 10^8 \pm 1.27 \times 10^{8*}$
	P	$3.40 \times 10^6 \pm 5.29 \times 10^{5*}$	$1.01 \times 10^9 \pm 8.14 \times 10^{7*}$
Salmonella Enteritidis		$2.20 \times 10^7 \pm 7.00 \times 10^{6*}$	$7.33 \times 10^8 \pm 7.51 \times 10^6$
Salmonella Enteritidis + raspberry preparations	W	$1.10 \times 10^7 \pm 9.50 \times 10^5$	$1.30 \times 10^9 \pm 1.27 \times 10^{8*}$
	S	$8.10 \times 10^6 \pm 1.71 \times 10^6$	$1.02 \times 10^9 \pm 6.43 \times 10^{7*}$
	P	$3.63 \times 10^6 \pm 1.19 \times 10^{6*}$	$1.08 \times 10^9 \pm 1.77 \times 10^{7*}$
Salmonella Typhimurium		$1.23 \times 10^7 \pm 6.03 \times 10^6$	$6.03 \times 10^8 \pm 1.20 \times 10^7$
Salmonella Typhimurium + raspberry preparations	W	$5.77 \times 10^6 \pm 1.08 \times 10^6$	$9.60 \times 10^8 \pm 1.64 \times 10^{8*}$
	S	$4.93 \times 10^6 \pm 2.10 \times 10^6$	$9.77 \times 10^8 \pm 6.11 \times 10^{7*}$
	P	$5.23 \times 10^6 \pm 2.44 \times 10^6$	$1.29 \times 10^9 \pm 6.51 \times 10^{7*}$
Control		$1.04 \times 10^7 \pm 8.14 \times 10^5$	$5.63 \times 10^8 \pm 1.05 \times 10^8$

EHEC – Enterohemorrhagic *E. coli*; W – raspberry seedless pomace preparation; S – seed preparation; P – native raspberry pomace preparation; *significant difference (p<0.05) compared to control.
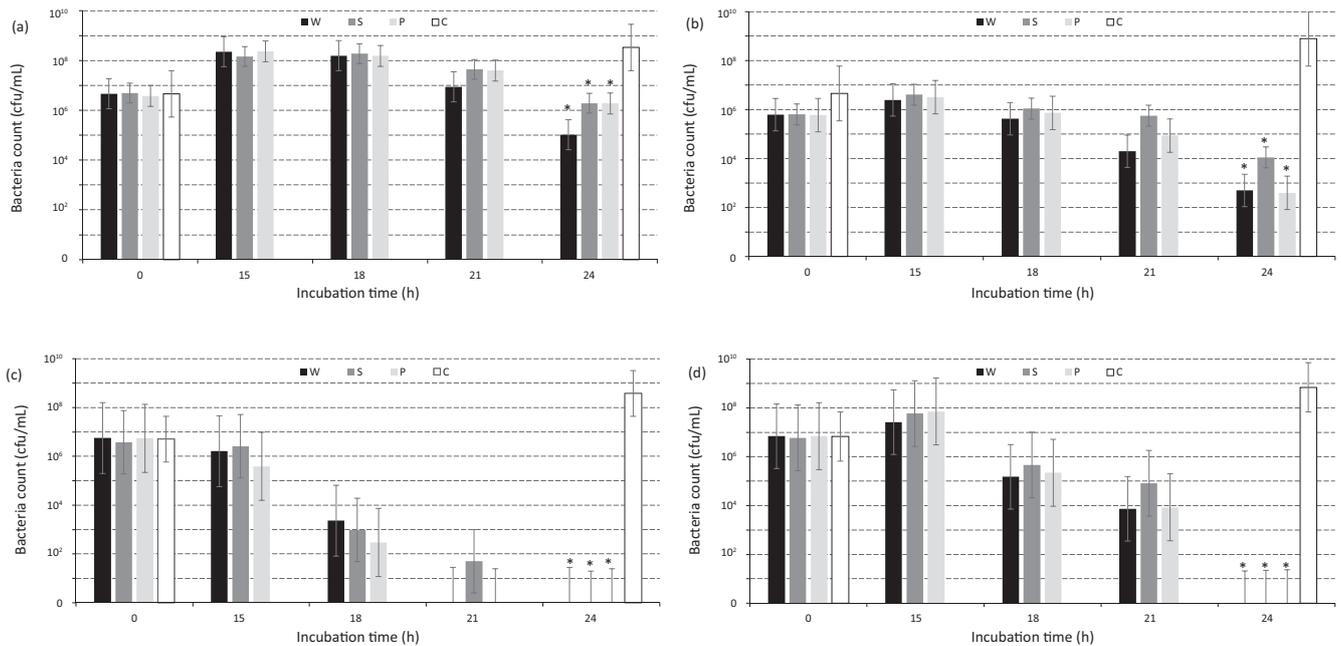
FIGURE 1. Count of the pathogenic bacteria (cuf/mL) in the co-culture with *L. rhamnosus* and raspberry seedless pomace, seeds and native pomace (W, S and P preparations, respectively) in the concertation of 2.0 mg/mL; (a) *Escherichia coli*, (b) *Listeria monocytogenes*, (c) *Salmonella* Enteritidis, and (d) *Salmonella* Typhimurium.

*Significant difference ($p < 0.05$) compared to control.

The co-culture with enteric pathogens did not influence the growth of lactobacilli during the 24-h incubation. Probiotic strain cell count was comparable to that observed in the control culture and approximated 10^8 cfu/mL. A significant increase in *L. rhamnosus* number was observed in all of the mixed cultures enriched with raspberry preparations. Their number after 24-h incubation ranged from 10^8 cfu/mL to 10^9 cfu/mL and exceeded the values obtained in the co-culture including bacterial strains only, regardless of preparation type (Table 4). A lack of the inhibitory effect of pathogenic bacteria on lactobacilli was also confirmed in the above-mentioned studies [Chen *et al.*, 2019; Adetoye *et al.*, 2018].

The enrichment of the pathogen-probiotic mixed culture with raspberry preparations resulted in the reduction of pathogen cell count (Figure 1). In most cases, the addition of raspberry preparations to the co-culture caused a greater reduction in pathogen populations, compared to cultures with *Lactobacillus* only. Similarly to the results obtained in the experiment with bacterial co-cultures not mixed with berry preparations, complete inactivation of the tested *Salmonella* strains was observed within in 24 h (Figure 1). Moreover, the complete elimination of *Salmonella* Enteritidis was reported after 21 h of incubation with *L. rhamnosus* mixed with W and P preparations. The concentration of *S.* Typhimurium cells in the mixed cultures decreased after 21 h from 10^6 cfu/mL to 10^3–10^4 cfu/mL. *L. monocytogenes* proved less sensitive to *L. rhamnosus* and raspberry preparations; however, the reduction in cell number from 10^5 to 10^2 cfu/mL was found after 24 h of the co-culture with the addition of pomace (P) and seedless pomace preparations (W). On the other hand, EHEC revealed the highest resistance to the mixed culture effect.

The results of the study showed no influence of the raspberry preparations type and chemical composition on their antimicrobial efficiency. The higher concentration of phenolics and ellagitannins in the seedless pomace preparation (W) did not result in its stronger inhibitory effect on enteric pathogens and *Lactobacillus*, compared to other raspberry preparations tested (S, P) (Table 2, 4 and Figure 1).

The results from the present study demonstrate that the combination of *Lactobacilli* and raspberry preparations was synergic against the pathogens tested. The idea of the coupled application of berry products and LAB or their metabolites was also examined in other studies. Terpou *et al.* [2019] used berries as an immobilization carrier of the probiotic lactobacilli in the functional frozen yogurt production, resulting in the improved product flavor and microbiological stability. In the study on the malolactic fermentation effect on the berry juice composition, Markkinen *et al.* [2019] found that fermentation by *Lactobacillus plantarum* decreased the content of hydroxycinnamic acids but not of anthocyanins in chokeberry. Finally, the high antimicrobial potential of natural formulations based on plant oils or extracts, and lactic acid against *L. monocytogenes*, *E. coli*, and *S.* Typhimurium was reported by Harich *et al.* [2018].

CONCLUSIONS

Our study showed no or low antibacterial effect of raspberry preparations against the bacteria tested. Although other researchers generally confirmed the inhibitory action of raspberry-based products on microorganisms, heterogeneous and contrary results were obtained. The differences in the antimicrobial potential of the tested raspberry products are probably

caused by the composition and amount of active compounds in the tested materials, or susceptibility of the bacterial strains tested. The coupled use of the probiotic *Lactobacillus* strain and raspberry preparations resulted in the synergic inhibitory effect against enteric pathogens. The obtained results suggest the possibility of the combined use of probiotics and active substances found in berries as natural antimicrobial agents for the food industry in functional food production. However, due to the high unpredictability of the final effect of these factors on bacteria growth, further studies in this research area need to be continued and extended.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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