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Antimicrobial Effects of Berries on *Listeria monocytogenes*

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Abstract

The purpose of this study was to first evaluate the antimicrobial effects of powder and extracts of berries (rose-hip, aronia, sea buckthorn and hawthorn) on the development of antibiotic-resistant *L. monocitogenes*. *Listeria monocytogenes* is considered one of the most important pathogens responsible for food-borne infection. Antimicrobial properties were evaluated using the standard Kirby-Bauer disk diffusion method. Bacterial inactivation networks were determined and compared, as well as the possibility of using powders and extracts of berries to control the risk of *Listeria monocytogees* infestation in the milk and dairy industry as well as in the meat industry. The effect of pH (4.78 - 4.43) and water activity (0.90 - 0.80) on the relationship between optical density (OD) at 600 nm and the plate count (CFU ml^{-1}) was investigated for *Listeria monocytogenes*. It was determined Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of berries for *L. monocytogenes*. The most relevant bacteriostatic and bactericidal effect on *L. monocytogenes* in the tested berries demonstrated sea buckthorn and rosehip.

Keywords

L. monocitogenes, Berries, Kirby-Bauer Test, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC)

1. Introduction

Listeria monocytogenes is considered one of the most important pathogens responsible for food-borne infection. It is often incriminated in outbreaks of human listeriosis [1] [2]. *Listeria monocytogenes* is a foodborne pathogen that can cause invasive severe human illness (listeriosis) in susceptible patients. Most

human listeriosis cases appear to be caused by consumption of refrigerated ready-to-eat foods [3]. In the European Union (EU), 1763 confirmed human cases of listeriosis were reported in 2013 by 27 member states. The EU notification rate was 0.44 cases per 100,000 population which represented an 8.6% increase compared to 2012 [4]. In 2013, there were 191 deaths caused by listeriosis in the EU. The highest number of fatal cases (64) was reported in France. Mortality rate in the EU was established at 15.6% among cases with known outcome. In 2013, a total of 13 outbreaks caused by *L. monocytogenes* were reported by seven MS and one non-MS. It was observed that the number of listeriosis in 2013 was slightly higher than in the previous years [5]. The European Food Safety Authority (EFSA) reported, in Europe, from 2008 to 2015, 37 food-borne outbreaks caused by *L. monocytogenes* that lead to 37 deaths [6]. Just from June of 2018, 47 cases have been reported, and nine patients have died due to or with the infection [7]. *L. monocytogenes* is an opportunistic bacterial pathogen that has the capacity to survive under extreme environmental conditions encountered in nature and in the food chain, such as high salt concentrations [8], large range of pH [9], desiccation [10] [11] and low temperatures [12].

In dairy industry, Listeria can contaminate directly or indirectly the products and the environment through contaminated raw milk, resulting in huge losses both in terms of public health and economy [2]. The presence of Listeria in yogurt may be a result of very bad quality of raw milk, inadequate heat treatment of milk or re-contamination as a result of using contaminated additives and poor hygiene during processing and packaging [13]. The pasteurization of milk has been recognized to ensure effective consumer safety against *L. monocytogenes* [14]. A study in Morocco [2] shows that out of 288 samples of dairy products examined, 17 (5.90%) were found to be contaminated with *L. monocytogenes*. Boubendir *et al.* [15] reported 5.76% prevalence of *L. monocytogenes* in bovine raw milk produced in the North Eastern Algeria. Similar results found by Guerra *et al.* [16], where the incidence of contamination was 5% in milk and dairy products sold in mainland Portugal. Gaya *et al.* [17] reported also a low incidence of 3.6% of *L. monocytogenes* in raw milk produced in Spain. Whereas in China, the prevalence of *L. monocytogenes* in raw milk is very low (0.23% to 1.2%) [18]. Benkerroum *et al.* [19] reported that bacteriocins produced by the lactic acid bacteria reduce counts of *L. monocytogenes* in cheese and yoghurt. Furthermore, several authors have confirmed that the growth or survival of *L. monocytogenes* in a food product depends on a variety of physico-chemical parameters, including pH, a_w and NaCl content [20] [21].

Listeria monocytogenes is a psychrotrophic microorganism (able to grow and multiply during cold storage) and even a few cells present in the final product can multiply to a level that is dangerous to consumers [22]. On the basis of literature data [23] [24], it can be concluded that *L. monocytogenes* has unfavourable conditions for growth in yogurt. However, these bacteria may survive in the final product for definite time depending on the type of product, its characteris-

tics (e.g. pH, competitive microflora), storage conditions (temperature) and other environmental circumstances. Previous studies have specifically shown that *L. monocytogenes* does not grow at a pH below 5.3 when the a_w is lower than 0.93 [25], or at a pH below 4.46 regardless of the a_w [8] [26]. In our study, the average physicochemical parameters associated with Lben and Jben are as such that they should limit (if not prevent) the growth of *L. monocytogenes*. The poor hygienic conditions during milking, transport, storage of milk and its use in the manufacture of Lben and Jben in traditional dairies, which do not respect the principles of food hygiene, can also be in favor of the contamination with *L. monocytogenes* [2]. When *L. monocytogenes* is present in a large number in raw milk some cells may survive the production process of yogurt and pose a serious hazard for consumers health [23]. Control of the feeding cattle, the general principles of food hygiene and milk pasteurization limit the contamination with *L. monocytogenes* [2]. Understanding the behavior of *Listeria monocytogenes* in fermented dairy products constitute sa crucial knowledge for Microbiological Risk Assessment (MRA) process, as well as Hazard Analysis and Critical Control Point (HACCP) system [23]. The experimental data regarding survival or inactivation of this foodborne pathogen during production and cold storage of yogurt at different temperatures can be described mathematically by predictive models [4] [27] [28] [29].

Predictive microbiology is based on the premise that the response of microorganisms to environmental factors is reproducible [4]. By defining the parameters that have the strongest effect on the behavior of microorganisms, it is possible to predict the response of microorganisms based on the performed observations [4] [30] [31]. *L. monocytogenes* is a Gram-positive, non-sporeforming foodborne pathogen, which is cold tolerant, widespread in the environment, and has the capability to grow under harsh conditions, including at elevated salt levels (up to 14%). Listeria has been known to persist in the food production environment for prolonged periods, despite regular sanitation [32]. The gram-positive bacterium *Listeria monocytogenes* is recognized as a food-borne pathogen with significance for humans [33], and major outbreaks of infection have been linked to the consumption of contaminated coleslaw [34], cheeses [35] and pasteurized milk [36]. The innate resistance of *L. monocytogenes* to many of the food preservation systems that are effective against other food-borne pathogens has prompted research aimed at developing combination systems for more effective control of this pathogen [37]. The purpose of the study was to determine the antibacterial activity of powder and extracts of berries (rose-hip, aronia, sea buckthorn and hawthorn) on *L. monocytogenes*.

2. Materials and Methods

Materials: extracts and powder berries—sea buckthorn, rose-hip, aronia and hawthorn. The test products were dissolved: 1 g produced in 4 ml of physiological solution, After which dilutions were made. Concentration of test

substances: 1 - 250 mg/ml; 2 - 125 mg/ml; 3 - 62.5 mg/ml; 4 - 31.25 mg/ml.

Initial concentration of preparations (liquids) of berries: sea buckthorn—0.12 mg/100ml; rose-hip—0.15 mg/100ml; aronia—0.18 mg/100ml; hawthorn—0.20 mg/100ml.

Product: Experimental samples (yogurt control and yogurt with addition (extracts and powder) of aronia were prepared in laboratory conditions in the Food Technology Department. Raw cow milk, obtained from local farm was received according to Governmental Decision No. 158, on 07.03.2019 with regard to the approval of the Technical Regulation "Milk and dairy products" that included technical conditions for the quality of raw cow milk collected for industrial processing [38]. The milk was pasteurized and standardized to a fat content of 2.5%. The milk was pasteurized (95˚C/30min), cooled to 45˚C and inoculated with 1.5% starter culture consisting of *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus salivarius spp. Thermophiles* (yogurt control) and yogurt with the addition (extracts and powder)—different concentrations of aronia. Notify the probe: Y—yogurt (control); YA 0.5—yogurt with 0.5% aronia powder; YA 0.75—yogurt with 0.75% aronia powder; YA 1.0—yogurt with 1.0% aronia powder.

Test strain: *L. monocytogenes* ATCC 19118; *Listeria monocytogenes* EGDe Mc Farland 0.5 (10^5) of *L. monocytogenes* strain ATCC 19118. Triptone Soya Broth (Oxoid) 6%, TSA—Triptone Soya Agar (Oxoid).

Methods: Antimicrobial Testing of extracts and powder berries

Well diffusion method:

In this study we used the agar diffusion procedure, called well diffusion method, it is a qualitative method used to determine antibacterial activity of the tested substances (extracts and powder from berries) on tested strain. Previously, the tested strain of listeria was spread with the swab on the surface of the plate. Then, 8 mm diameter wells were made in the Mueller Hinton agar plate. The dissolved extracts were introduced into each well. If the bacterial strain is susceptible to the antimicrobial agent, then a transparent area is observed around the well. This area represents the inhibition zone of growth. If the bacteria is resistant to the antimicrobial agent around the well will be observed growth. The sensitivity or resistance of bacteria were calculated by measuring the inhibition zone diameter around the wells. The antimicrobial activity is calculated in millimeter: the total diameter of growth inhibited zone minus diameter of the well [39].

1 g of yogurt was added 1 - 2 drops of microbial suspension according to the Mc Farland 0.5 (10^5) turbidity standard from *L. monocytogenes strain ATCC 19118*. The infected samples were incubated in thermostat at 37˚C for 24, 48 hours and respectively 15 days.

Identification of minimum inhibitory concentration (MIC) The methodology for this experiment is based on the work of Lambert and others [40]. Ten μL of inoculum were dispensed in each well. After inoculation, the plate was incubated at 25˚C for 24 h and the optical density (O.D.) of each well was recorded at 600 nm every 20 min after shaking [41].

Identification of minimum bactericidal concentration (*MBC*)

The methodology described below is an adaptation of the Minimum Bactericidal Concentration Testing from the Clinical Microbiology Procedures Handbook [42]. *L. monocytogenes* was cultured on tryptone soya (TS) agar plate at 37˚C for 18 h, and a cellular suspension was prepared in sterile distilled water.

3. Results and Discussions

Listeria monocytogenes is considered one of the most important pathogens responsible for foodborne infection. It is often incriminated in outbreaks of human listeriosis [1]. A number of studies have demonstrated that *L. monocytogenes* is more acid tolerant than most food-borne pathogens, although the sensitivity of the organism to organic acids varies with the nature of the acidulant used [43], *Listeria spp*. prefers to grow at pH 7 -8 but they will grow in the range pH 5 - 10 and may survive and grow in material with a pH as low as 4.4 (**Table 1**). Results of another study demonstrate a high risk associated with consumption of bulked raw milk and fermented dairy products in due to occurrence of *Listeria spp*. [44].

In previous studies [46] [47] it has been found that sea buckthorn, rose-hip, aronia and hawthorn have antimicrobial effects on pathogenic microorganisms. Following the tests, the authors established that the additives of rose and hawthorn in the sausage recipe can control the growth rate of microorganisms, including pathogens. By studying the Lag and Logarithmic growth phases of pathogenic microbial strains we determined that the hawthorn has a greater bacteriostatic effect on strains of *S. aureus ATCC 25923* and *E. coli ATCC 25922* and the rose-hip has a greater bacteriostatic effect on *Salmonella strains Abony ATCC 6017, Klebsiella pneumoniae ATCC 13883*. The most relevant antimicrobial effect was seen for berry powders on *E. coli* strains inoculated in tested cream cheese samples. The additions of rose-hip and aronia powders manifested major antimicrobial effect on *Salmonella* strains. The addition of hawthorn powder manifested major antimicrobial effect on *Staphylococcus aureus* [46].

The results of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for *L. monocitogenes* strain are presented in **Table 2**, **Table 3**. The results of the study showed that aronia and hawthorn have no antimicrobial activity against *L. monocytogenes*. Significant positive results showed the extracts and powders of sea buckthorn and rose-hip.

It has been studied whether berries have bactericidal effect on *L. monocytogenes* in food (yogurt). Yogurt samples (I) were infected with *L. monicytogenes*, the initial concentration being 10^5 cells. After 24, 48 hours a decrease in bacteria was found in all samples, including in the control sample, without the addition of berries. After 15 days the samples were tested and it was found that *L. monocytogenes* was not present (**Table 4**). In the samples where additions of berries (extracts and powders) were introduced, the rate of reduction was much higher. The bactericidal effect of berries has been demonstrated.

Table 1. Factors identified to have an impact on the growth and survival of *L. monocytogenes*[a](adapted from SANCO [45]).

Factor	Lower Growth Limit	Optimum[b]	Upper Growth Limit	Can Survive[c] (No Growth)
Temperature (˚C)	−1.5 to +3.0	30.0 to 37.0	45.0	−18.0
pH[d]	4.2 to 4.3	7.0	9.4 to 9.5	3.3 to 4.2
Water Activity (a_w)	0.90 to 0.93	0.99	>0.99	<0.90
Salt Concentration [e](%)	<0.5	0.7	12 - 16	≥20
Atmosphere	Facultative anaerobe (it can grow in the presence or absence of oxygen, e.g. in a vacuum or modified atmosphere package)			
Heat Treatment during Food Processing	A temperature/time combination e.g. of 70˚C and 2 min is required for a D6 (*i.e.* 106 or 6 decimal) reduction in numbers of *L. monocytogenes* cells. Other temperature/time combinations may also provide the same reduction			

[a]Based on experimental data and hence provide only a rough estimate; [b]Optimum indicates when the growth of *L. monocytogenes* is fastest; [c]Survival period will vary depending on nature of food and other factors; [d]Inhibition of *L. monocytogenes* is dependent on type of acid present; [e]Based on percent sodium chloride, water phase.

Table 2. MIC and MBC *L. monocytogenes* ATCC 19118.

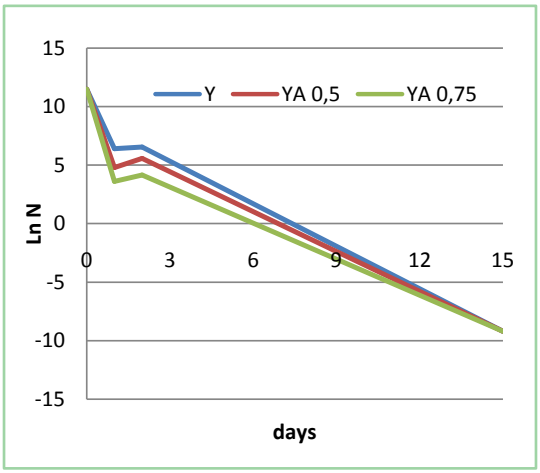
Nr.	Berries	Diameter of the complete growth inhibition zone (mm)			
		250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml
1.	Sea buckthorn (*extract*)	22.5 ± 0.25	19.25 ± 0.22	14.25 ± 0.22	10.75 ± 0.22
2.	Sea buckthorn (*powder*)	16.33 ± 0.26	12.5 ± 0.25	10.75 ± 0.22	0.0 ± 0.0
5.	Rose-hip (*extract*)	16.33 ± 0.26	12.25 ± 0.22	10.25 ± 0.22	0.0 ± 0.0
6.	Rose-hip (*powder*)	17.75 ± 0.42	15.75 ± 0.22	11.75 ± 0.22	0.0 ± 0.0

Table 3. The diameter of the complete growth inhibition zone of different species of *L. monocytogenes* under the action of the extract and powder of sea buckthorn rose-hip.

Nr.	Berries	*L. monocytogenes* ATCC 19118	Berries	*L. monocytogenes* EGDe
		Diameter of the complete growth inhibition zone (mm)		Diameter of the complete growth inhibition zone (mm)
		250 mg/ml		250 mg/ml
1.	Sea buckthorn (*extract*)	22.25 … 22.75	Sea buckthorn C_1, C_2 (concentrated hydroalcoholic extract)	30 … 32
2.	Sea buckthorn (*powder*)	16.07 … 16.59	Sea buckthorn H_1, H_2 (hydroalcoholic extract)	29 … 30
5.	Rose-hip (*extract*)	16.07 … 16.59	Rose-hip C_1, C_2 (concentrated hydroalcoholic extract)	20 … 21.5
6.	Rose-hip (*powder*)	17.33 … 18. 17	Rose-hip H_1, H_2 (hydroalcoholic extract)	22 … 22

Table 4. The influence of different concentrations of aronia on the bactericidal effect (*L. monocitogenes*) in yogurt.

The product tested	*L. monocytogenes, ln N*			
	initially infected with bacteria	**1 day**	**2 days**	**15 days**
Y	11.51	6.40	6.55	−9.21
YA 0.5	11.51	4.80	5.58	−9.21
YA 0.75	11.51	3.61	4.16	−9.21
YA 1.0	11.51	5.58	5.24	−9.21
YA 0.5	11.51	4.88	3.61	−9.21
YA 0.75	11.51	5.46	4.30	−9.21
YA 1.0	11.51	5.02	2.20	−9.21



Results have been obtained that have been reported in other research studies. The main mechanism of bactericidal effect of yogurt on foodborne pathogens seems to be the decline in pH due to lactose fermentation by *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus salivarius ssp. thermophilus* added to milk as a starter culture, as well as the production of organic acids, mainly lactic acid [4] [48] [49]. The researchers found that lag phase and growth rate of *Listeria* were affected not only by storage temperature but also by lactic acid concentration and pH of product ranged from 5.2 do 5.8. J. Kowalik *et al.* [50] observed more dynamic growth of *L. monocytogenes* in milk samples, having a pH of 6.6, and stored under conditions identical to those applied for the samples of the cottage cheese. Some authors have suggested that antimicrobial activity of yogurt is not exclusively due to accumulation of lactic acid and may be also the effect of lactic acid and other compounds such as hydrogen peroxide, carbon dioxide, acetaldehyde, polysaccharide and bacteriocins [51]. The pH value of 4.6 is usually considered as a minimum pH permitting the growth of *L. monocytogenes* in food [52]. However, pH minimum as low as 4.39 has also been reported [53]. Low pH of the environment is an important factor responsible for the re-

duction of Listeria population in yogurt, the correlation between the number of *L. monocytogenes* and pH amounted to 0.832 [4]. Listeria is one of the few foodborne pathogens that can multiply at low water activity. Studies have shown that this organism can multiply at a_w below 0.93 [54] [55].

In the food industry, salting is a food preservation method designed primarily to obtain lower water activity. However, *L. monocytogenes* is able to survive high concentration of salt and is thus not an easy pathogen to control by osmotic stress alone. Osmo-adaptation in bacteria can involve both physiological changes and as well as regulation at the gene expression level [56].

The low counts observed in Bongo may be attributed to the low pH and other antimicrobial compounds such as bacteriocins produced by lactic acid bacteria. Also, the type of acid and the storage temperature have a marked effect on the ability of *Listeria* to survive and grow at low pH. On the other hand, the presence of *Listeria spp*. in Bongo may be attributed to contamination from raw milk, the starter culture inoculum and slow rate of acid formation and pH decline [44].

A number of studies have demonstrated the inhibitory activity of organic acids against *L. monocytogenes* and have shown that the effects are mainly related to the amount of undissociated acid [57] [58] [59]. Benzoate and formate (used alone) they were highly effective at killing *L. monocytogenes* at pH 3. Nevertheless, in all cases addition of ethanol resulted in shorter killing times. The most effective bactericidal combination, 5% ethanol and 50 mM formate, resulted in 5 log units of killing in just 4 min [43]. A number of studies have demonstrated the inhibitory activity of organic acids against *L. monocytogenes* and have shown that the effects are mainly related to the amount of undissociated acid [57] [58] [59]. The most common way to assess microbial growth in solution is the measurement of the optical density at 600 nm, or short OD_{600}. The method is based on absorbance detection mode and basically determines which portion of light passes through a sample, more specifically through a suspension of microorganisms [60]. The calibration lines obtained by plotting log OD_{600nm} values vs. log plate counts showed high determination coefficients for all the tested strains in the range of OD_{600nm} values 0.1 - 1.5 corresponding to approximately 10^6 - 10^9 CFU/ml [61].

A case study was performed to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentrations (MBC) of the extracts and powders of the dogwood and mulberry on *L. monotogenes*. The results of the study are presented in **Table 5**.

The results of the study showed that hydroalcoholic extracts of sea buckthorn and rosehip showed beneficial results on the inhibitory and bactericidal effect of *L. monocytogenes*. The sea buckthorn has shown antimicrobial properties for *L.monocytogenes* more pronounced than rose-hip. Probably these properties are due to the chemical composition of sea buckthorn and rosehip. These berries are rich in organic acids. Determining the chemical composition of the rosehip, used in the case study, we found that the fruit contains: malic acid—154.3; citric

Table 5. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) for *L. monocytogenes EGDe*.

The tested material	Sea buckthorn		Rose-hip	
	OD, Λ_{600}	*Minimum Inhibitory Concentration* (*MIC*), *mg/ml*	OD, Λ_{600}	*Minimum Inhibitory Concentration* (*MIC*), *mg/ml*
C_1	0.071	2.6	0.059	4.2
C_2	0.047	5.2	0.053	16.7
H_1	0.061	2.6	0.020	4.2
H_2	0.072	2.6	0.024	4.2

C_1, C_2—concentrated hydroalcoholic extract; H_1, H_2—hydroalcoholic extract; DO—optical density.

acid—1684; succinic acid—56.89; lactic acid—30.54; acetic acid—23.85, including L-ascorbic acid—72 mg/100 g s.u. Data recorded over the world, over time, show that dried rose hips contain ascorbic acid between 0.1%, 0.5% and 1.0%, and some varieties even up to 9% [62].

In the study conducted by Țifrea Anca it was found that the addition of sea buckthorn pulp positively influences the increase of lactic acid content in yogurt [63]. The increase in lactic acid content may be due in part to the addition of sea buckthorn pulp due to the content of organic acids in it. Compared with the control sample which initially had 4.6 and finally 4.2 it can be seen that the addition of sea buckthorn influenced the pH by a greater decrease in the samples with higher addition of sea buckthorn in a similar way to acidity.

Another factor that would control the microbiological risk in food is water activity. Most bacterial species, including *L. monocytogenes* grow optimally at a water activity (a_w) of 0.97 [45] [64]. *Listeria monocytogenes* is one of the few food-borne pathogens that can grow at an a_w value below 0.93 [65]. However, *L. monocytogenes* also has the ability to grow to a level of a_w 0.90 [66]. In our case study it was found that in yogurt with the addition of powder rose hip the value a_w was in the range of 0.876 … 0.877. The reduction in aw was also found in the samples of yogurt with the addition of sea buckthorn powder, compared to the control samples. This can probably be explained by the fact that bactericide was more prominent in yogurt with the addition of berries compared to classic yogurt. The cumulative effect of the quantitative and qualitative content of acids in the composition of sea buckthorn and rosehip extracts and powders and the property of reducing the pH and activity of water, make berries have bacteriostatic and bactericidal properties on *Listeria monocytogenes*.

4. Conclusions

Based on the study, sea buckthorn and rose-hip (extract and powder) were found to be excellent remedies for controlling the risk of *L. monocytogenes* infestation of food. This is due to the cumulative effect of the chemical composition of these berries (antioxidant content, organic acids, etc.), increased acidity,

reduced pH and water activity of the food environment in which they were introduced below the development values of *L. monocytogenes*.

The minimum dose of inhibition and the minimum bactericidal dose of berries were determined. The use of added berry powders in the recipe for the production of dairy products can have two meanings: improving the nutritional value of the food and increasing the product shelf-life by keeping the microbiological risk under control, including *L. monocytogenes*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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