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Combination of phenolic acids and essential oils against *Listeria monocytogenes*

Lye Miyague [a], Renata E.F. Macedo [a], Giuseppe Meca [b], Richard A. Holley [c], Fernando B. Luciano [a, *]

[a] School of Agricultural Sciences and Veterinary Medicine, Pontifícia Universidade Católica do Paraná, São José dos Pinhais, 83010-500, Brazil
[b] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100, Burjassot, Spain
[c] Department of Food Science, Faculty of Agricultural and Food Sciences, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

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ABSTRACT

Listeria monocytogenes is a psychrotrophic pathogen widely distributed in meat processing plants. Listeriosis presents very low morbidity, but very high mortality rates. Several outbreaks involving this bacterium have been reported due to the consumption of refrigerated meat and dairy products. The objective of the present study was to evaluate the *in vitro* antimicrobial activity of 10 phenolic acids (PAs) and 4 essential oil compounds (EOCs) as natural alternatives to control *L. monocytogenes* growth. The minimum inhibitory concentration (MIC) of all natural compounds was determined at pH 5, 6 and 7. The 4 most potent PAs (ρ-hydroxybenzoic acid, ferulic acid, o-coumaric acid and syringic acid) were selected and tested in combination with the 2 most active EOCs (allyl isothiocyanate and carvacrol), giving a total of 8 combinations. Lower pH levels enhanced the antimicrobial activity of both PAs and EOCs, with the exception of geraniol, which had the same MIC at all pH levels. Most combinations of essential oils and phenolic acids showed synergistic effects at pH 5, whereas only ferulic acid plus allyl isothiocyanate showed synergism at pH 6. The combined use of these natural compounds can improve their antimicrobial activity and may eliminate *L. monocytogenes* from mildly acid food products.

1. Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, non-spore forming and motile bacteria (Gray & Killinger, 1966). Among all species of *Listeria*, *L. monocytogenes* is the only one consistently pathogenic to humans and it is usually transmitted through contaminated foods (Kathariou, 2002). This bacterium is widely distributed in meat processing plants, and many listeriosis outbreaks have been reported due to the consumption of ready-to-eat (RTE) meat products (Barmpalia et al., 2005; Couture, 2009).

Different from most pathogenic bacteria, *L. monocytogenes* can survive and grow on refrigerated foods. Moreover, *L. monocytogenes* can grow aerobically or anaerobically at pH levels varying from 4.6 to 9.4 (Nørrung, Andersen, & Buncic, 2009). Consequently, many refrigerated vacuum packaged meat products, with extended shelf-life, can support the growth of *L. monocytogenes*. The application of high temperature to these products can destroy the pathogen, but contamination usually occurs after cooking and before packaging (Sofos & Geornaras, 2010). The United States Department of Agriculture (USDA) has adopted a zero-tolerance policy for the pathogen in RTE meats (FDA, 2003). However, the European Commission (EC, 2005) and Health Canada (HC, 2011) have established a limit of 2 Log CFU g^{-1} of *L. monocytogenes* in meat products where this bacterium cannot grow (e.g. salami). Therefore, there is a great interest of the food industry on methods that prevent the growth or kill this pathogen in RTE meats.

In 2008, Maple Leaf Foods was involved in one of the worst food outbreaks in Canadian history. About 200 products that were manufactured at their meat processing facility in Toronto were recalled. In addition, there were 57 confirmed cases of listeriosis and 23 people died. The economic loss incurred by Maple Leaf Foods surpassed US$ 50 million, and involved costs of the recall itself, plant closures, product loss, lawsuits and loss of the market share (Greenberg & Elliott, 2009). From an analysis of these numbers, the urgent need to minimize the risk of food contamination becomes clear. However, consumers persistently request products that are minimally processed which maintain their fresh flavor and have extended shelf-life (Burt, 2004). Hence, use of

* Corresponding author. Tel.: +55 41 3299 4436; fax: +55 41 3299 4423.
E-mail address: fernando.luciano@pucpr.br (F.B. Luciano).

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natural preservatives that can prevent microbial growth in foods is a popular industry trend (Schuenzel & Harrison, 2002; Skandamis & Nychas, 2002). Some of the most studied natural antimicrobials are essential oils (EO) (Burt & Reinders, 2003). These compounds act at the plasma membrane, leading to the leakage of cytoplasmic content and subsequent cell death. However, most of these compounds significantly change the sensory aspects of meat products when added at levels that are effectively antimicrobial (Burt, 2004). Thus, the utilization of other natural substances that could potentiate the antimicrobial activity of EOs is of great interest to the meat industry.

The antimicrobial activity of phenolic acids (PA) towards *L. monocytogenes* has been reported (Apostolidis, Kwon, & Shetty, 2007; Klancnik, Piskernik, Jersek, & Mozina, 2010). These compounds work best at lower pH levels, when they are not ionized and can easily cross the plasma membrane. Once in the bacterial cytoplasm, these compounds dissociate, decreasing the internal pH. This leads to the inactivation of many metabolic pathways.

Although the anti-listerial activity of PAs and EOs is well established, there is no report in the literature describing their activity in combination. The objective of the present study was to evaluate the antimicrobial activity of phenolic acids and essential oil compounds (EOCs) alone and in combination against *L. monocytogenes*.

2. Materials and methods

2.1. Microorganisms and culture media

L. monocytogenes ATCC 19117 was donated by Dr. Cleide R. W. Vieira from the Food Science and Technology Center of the Federal University of Santa Catarina, Florianópolis, Brazil. Bacteria were maintained at − 80 °C prior to the experiments. Then, *L. monocytogenes* were reactivated in brain-heart infusion (BHI) broth and incubated at 30 °C for 24 h. The organism was re-inoculated in BHI broth with pH adjusted to 5, 6 or 7 for 16 h at 30 °C, to allow bacterial adaptation to the challenge pH values before the Minimum Inhibitory Concentration (MIC) tests were conducted. *L. monocytogenes* was transferred (0.1 ml in 9.9 ml of BHI broth) again to fresh broth at pH 5, 6 or 7 and incubated at 30 °C until mid-exponential growth was reached (~10^8 CFU/ml; 0.5 McFarland scale).

2.2. Determination of the minimum inhibitory concentration

The anti-listerial effect of 10 PAs and 4 EOCs (Table 1) at three pH levels (5, 6 and 7) was tested. All substances were diluted in dimethyl sulfoxide (maximum of 2% in the final solution) to facilitate their solution in the liquid media. Treatments were performed in screw-capped tubes by adding 9.9 ml of Brain Heart Infusion (BHI) broth + antimicrobial agent and 100 μL of *L. monocytogenes* ATCC 19117 inoculum at mid-exponential phase (~10^8 CFU/mL). Control groups contained 9.9 ml BHI broth, 2% dimethyl sulfoxide and 100 μL bacterial inoculum. Then, tubes were incubated for 24 h at 30 °C (pH 6 and 7) or for 48 h at 30 °C (pH 5). The Minimum Inhibitory Concentration (MIC) was considered as the lowest dose where no increase in optical density (600 nm) was observed (CLSI, 2012).

Table 1
Natural antimicrobials tested against *L. monocytogenes*.

Phenolic acid	Essential oil
ρ-Hydroxybenzoic acid	Allyl isothiocyanate
Cinnamic acid	Carvacrol
Ferulic acid	Geraniol
4-Coumaric acid	Cinnamaldehyde
Syringic acid	
Vanillic acid	
o-Coumaric acid	
Sinapic acid	
Gallic acid	
Protocatechuic acid	

Table 2
Minimum inhibitory concentration of phenolic acids against *Listeria monocytogenes* growth in BHI broth.

Phenolic acid	MIC (mM)		
	pH 5	pH 6	pH 7
ρ-Hydroxybenzoic	5	5	10
Cinnamic	5	10	20
Ferulic	2.5	5	10
4-Coumaric	5	5	15
Syringic	5	5	10
Vanillic	5	5	10
o-Coumaric	1.25	5	10
Sinapic	5	5	10
Gallic acid	5	10	ND[a]
Protocatechuic	5	10	20

[a] ND – MIC not detected using doses up to 20 mM.

Table 3
Minimum inhibitory concentration of essential oils against *Listeria monocytogenes* growth in BHI broth.

Essential oil	MIC (mM)		
	pH 5	pH 6	pH 7
Allyl isothiocyanate	0.27	0.51	1.02
Carvacrol	0.65	1.63	1.63
Cinnamaldehyde	2.0	4.0	4.0
Geraniol	2.85	2.85	2.85

2.3. Inhibitory effect of PAs and EOCs in combination

Based on their MIC values, 4 phenolic acids (ρ-hydroxybenzoic acid, ferulic acid, o-coumaric acid and syringic acid) and two essential oil compounds (allyl isothiocyanate, AITC, and carvacrol) were selected for use in combination against *L. monocytogenes*. The combined antimicrobial solution consisted of one phenolic acid and one essential oil dissolved in 2% DMSO, giving a total of 8 test combinations (Table 4). The concentrations of the natural antimicrobials started from their MIC and were serially diluted in two-fold steps. Fractional inhibitory concentrations (FICs) for each PA + EOC combination were calculated using the following equations: FIC of EOC = MIC EOC in combination/MIC of EOC alone; FIC of PA = MIC of PA in combination/MIC of PA alone. An FIC_{index} = FIC of EOC + FIC PA was also calculated to assess the type of antimicrobial interaction between the test compounds (Palaniappan & Holley, 2010). An

Table 4
Fractionary Inhibitory Concentration index of EOs in combination with PAs against *Listeria monocytogenes* ATCC 19117.

Essential oil	Phenolic acid			
	o-Coumaric acid	Ferulic acid	ρ-Hydroxybenzoic acid	Syringic acid
AITC pH 5	0.5	0.5	0.5	0.75
Carvacrol pH 5	0.5	0.5	0.75	0.75
AITC pH 6	0.75	0.5	1.0	1.0
Carvacrol pH 6	1.0	1.0	1.0	1.0

An $FIC_{index} \leq 0.5$ indicates synergy. An $FIC_{index} > 0.5$ and < 4.0 indicates an insignificant interaction. An $FIC_{index} \geq 4.0$ represent an antagonistic interaction.