# COMBINATION OF PHENOLIC ACIDS AND ESSENTIAL OILS AGAINST *LISTERIA MONOCYTOGENES*

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**Abstract – *Listeria monocytogenes* is a psycrotrophic pathogen widely distributed in meat processing plants Listeriosis presents very low morbitity, but very high mortality rates. Several outbreaks involving this bacterium have been reported due to the consumption of refrigerated meat products. The objective of the present study was to evaluate the antimicrobial activity *in vitro* of 10 phenolic acids and 4 essential oils as natural alternatives to control *L. monocytogenes* in meat products. The minimum inhibitory concentration (MIC) of all natural compounds was determined at pH 5, 6 and 7. The 4 most potent phenolic acids were selected (ρ-hydroxybenzoic acid, ferulic acid, o-coumaric acid and syringic acid) and tested in combination with the 2 most active essential oils (allyl isothiocyanate and carvacrol), giving a total of 8 combinations. Lower pH levels favored the antimicrobial activity of both phenolic acids and essential oils, with the exception of geraniol, which presented the same MIC at all pH levels. All combinations of essential oils and phenolic acids presented synergistic effect at pH 5, whereas only ferulic acid plus allyl isothiocyanate or carvacrol showed synergism at pH 6. Potentially, the combined utilization of these natural antimicrobials could optimize their utilization to eliminate *L. monocytogenes* in meat products.**

**Key Words – Listeriosis, Natural antimicrobials, Synergism**

- INTRODUCTION

*Listeria monocytogenes* is a Gram-positive rod, non-spore forming and motile [1]. Among all species of *Listeria*, *L. monocytogenes* is the only one that is pathogenic to humans [2], and its source of transmission is usually through contaminated foods [3]. 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 [4-5].

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 [6]. Consequently, many refrigerated vacuum packaged meat products, with extended shelf-life, can support the growth of *L. monocytogenes*. The application of high temperature in these products can destroy the pathogen, but the contamination usually occurs after the cooking process and before packaging [7]. The United States Department of Agriculture (USDA) has adopted a zero-tolerance policy to the pathogen in RTE meats [8]. However, the European Comission [9] and Health Canada [10] established a limit of 2 Log CFU/g of *L. monocytogenes* in meat products where this bacterium cannot grow (e.g. salami). Therefore, there is a great interest of the 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 worse food outbreaks of 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 expenses towards Maple Leaf Foods surpassed US$ 50 million, and involved recall process, product loss, lawsuits and losses of the market share [11]. Analyzing these numbers, it is clear the urgent need to minimize the risk of food contamination. However, consumers are requiring products mildly processed which maintain their fresh

flavor and have extended shelf-life [12]. Hence, use of natural preservatives that could avoid microbial growth in foods is a current trend [13-14]. Some of the most studied natural antimicrobials are essential oils (EO) [15]. These compounds act at the plasmatic membrane, leading to the leakage of cytoplasmic substrates, resulting in cell death. However, most of these compounds significantly change the sensory aspects of meat products when added at efficient antimicrobial doses [12].
Thus, the utilization of other natural substances that could enhance the antimicrobial activity of EO is of great interest for the meat industry. The antimicrobial activity of phenolic acids (PA) towards *L. monocytogenes* has been reported [16-17]. These compounds work best at lower pH levels, when they are at their molecular form and can easily cross the plasmatic membrane. Once in the cytoplasm, these compounds dissociate, decreasing the bacterial internal pH. This leads to the inactivation of many metabolic pathways.

Although the anti-listerial activity of PA and EO is well established, there is no report in the literature about their activity in combination. The objective of the present study was to evaluate the antimicrobial activity of phenolic acids and essential oils alone and in combination against *L. monocytogenes*.

- MATERIALS AND METHODS

The anti-listerial effect of 10 PA and 4 EO (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 homogeneization in the liquid media. Treatments were performed in screw-capped tubes by adding 9.9 mL of BHI broth + antimicrobial agent and 100 µL of *L. monocytogenes* ATCC 19117 inoculum at mid-exponential phase (~$10^8$). Control groups contained 9.9 of BHI broth, 2% dimethyl sulfoxide and 100 µL bacterial inoculum. Then, tubes were incubated for 24 h at 35°C (pH 6 and 7) or for 48 h at 35°C (pH 5). The Minimum Inhibitory Concentration (MIC) was considered as the lowest dose where no increase in optical density (600 nm) was observed.

Four phenolic acids (ρ-hydroxybenzoic acid, ferulic acid, o-coumaric acid and syringic acid) and two essetial oils (allyl isothiocyanate and carvacrol) were selected, based on their MIC, to be used in combination against *L. monocytogenes.* The combined antimicrobial solution consisted of one phenolic acid and one essential oil dissolved in DMSO, giving a total of 8 combinations. When combined, the natural compounds were added to the final solution as follows: Dose 1 = MIC EO + MIC PA; Dose 2 = ½ MIC EO + ½ MIC PA; Dose 3 = ¼ MIC EO + ¼ MIC PA; Dose 4 = $^1/_8$ MIC EO + $^1/_8$ MIC PA; Dose 5 = ¼ MIC EO + ½ MIC PA; and Dose 6 = $^1/_8$ MIC EO + ½ MIC PA. The combinations were tested only on pH 5 and 6 since both EO and PA presented better activity at lower pH levels.

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

| Phenolic Acid | Essential Oil |
|---|---|
| ρ-Hidroxybenzoic acid | Allyl Isothiocyanate |
| Cinnamic acid | Carvacrol |
| Ferulic acid | Geraniol |
| 4-Coumaric acid | Cinnamaldehyde |
| Syringic acid | |
| Vanillic acid | |
| o-Coumaric acid | |
| Sinapic acid | |
| Gallic acid | |
| Protocathecuic acid | |

- RESULTS AND DISCUSSION

As expected, most antimicrobials showed stronger antimicrobial activity at lower pH (Table 2 and 3). The only exception was geraniol, which presented the same anti-listerial response at all pH tested. Although *L. monocytoges* is resistant to acid pH, lower levels of pH work as a hurdle against the growth of this pathogen. Moreover, phenolic acids were shown to have higher antimicrobial activity when at pH values that were close to their pKa [18], which is usually around 4.5-4.9.

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

| Phenolic acid | MIC | | |
|---|---|---|---|
| | pH 5 | pH 6 | pH 7 |
| ρ-Hydroxybenzoic | 5mM | 5mM | 10mM |
| Cinnamic | 5mM | 10mM | 20mM |
| Ferulic | 2.5mM | 5mM | 10mM |
| 4-Coumaric | 5mM | 5mM | 15mM |
| Syringic | 5mM | 5mM | 10mM |
| Vanillic | 5mM | 5mM | 10mM |
| o-Coumaric | 1.25mM | 5mM | 10mM |
| Sinapic | 5mM | 5mM | 10mM |
| Gallic acid | 5mM | 10mM | ND* |
| Protocathecuic | 5mM | 10mM | 20mM |

*ND – MIC not detected using doses up to 20 mM.

Among all PA, o-coumaric acid acid showed the strongest antimicrobial activity overall. Cinnamic, gallic and protocathecuic acids presented the poorest antilisterial activities overall, whereas all other PA showed similar effect. Therefore, the 4 PA selected for the next step of this study were o-coumaric acid (o-CA) - based on antimicrobial activity -, ferulic (FA), ρ-hydroxybenzoic (ρ-HBA) and syringic acids (SA) – based on activity and cost.

Allyl isothiocyanate (AITC) presented the strongest anti-listerial activivity, followed by carvacrol (Carv), cinnamaldehyde and geraniol (Table 3). Thereby, AITC and Carv were chosen to be used in combination with PA against *L. monocytogenes*.

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

| Essential oil | MIC | | |
|---|---|---|---|
| | pH 5 | pH 6 | pH 7 |
| Allyl isothiocyanate | 0.27 mM | 0.51 mM | 1.02 mM |
| Carvacrol | 0.65 mM | 1.63 mM | 1.63 mM |
| Cinnamaldehyde | 2 mM | 4 mM | 4 mM |
| Geraniol | 2.85 mM | 2.85 mM | 2.85 mM |

The combination of EO and PA was used to evaluate the possibility of synergism and/or additive effect among these compounds. A total of 8 combinations were tested as described on session II. Results are presented on Table 4.

Table 4 Anti-listerial effect of essential oils and phenolic acids used in combination

| | Phenolic Acid | | | |
|---|---|---|---|---|
| Essential oil | o-CA | FA | ρ-HBA | SA |
| AITC pH 5 | Dose 5** | Dose 5 | Dose 5 | Dose 5 |
| Carv pH 5 | Dose 5 | Dose 5 | Dose 5 | Dose 5 |
| AITC pH 6 | Dose 2* | Dose 5 | Dose 2 | Dose 2 |

| | | | | |
|---|---|---|---|---|
| Carv pH 6 | Dose 2 | Dose 2 | Dose 2 | Dose 2 |

*Dose 2 is ½ MIC EO + ½ MIC PA
**Dose 5 is ¼ MIC EO + ½ MIC PA

Most combinations at pH 6 presented only additive effect. The only exception was the use of AITC coupled with FA, which showed synergistic effect (¼ MIC AITC + ½ MIC FA). In addition, all combinations at pH 5 showed synergistic effect, where the EO could be reduced to ¼ of its MIC. Potentially, this technology could have great application for the food industry since the strong taste of EO is the main drawback of their utilization as food preservatives [12]. The complimentary mechanism of action of EO and PA certainly contributed to the potentialization of their antimicrobial activity.

To our knowledge, this is the first report showing the synergistic effect between carvacrol/AITC and phenolic acids against a foodborne pathogen.

- CONCLUSION

Allyl isothiocyanate and carvacrol showed the strongest activity against *L. monocytogenes* among the EO tested, whereas o-coumaric acid, ferulic, ρ-hydroxybenzoic and syringic acids were the most potent PA. All combinations of EO and PA showed synergistic effect at pH 5, and AITC + FA also presented synergism at pH 6. Acidic solutions containing these compounds could reduce the levels of *L. monocytogenes* in meat products without causing significant alterations on their sensory characteristics. Further studies will evaluate the application of AITC and FA on the surface of frankfurters and cooked ham in order to eliminate or reduce the contamination of *L. monocytogenes*.

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REFERENCES

- Franco, B. D. G. M. & Landgraf, M. (1996). Microbiologia dos Alimentos. Rio de Janeiro: Editora Atheneu.
- Cesar, A. P. R., Mesquita, A. J., Prado, C. S., Nunes, I. A. & Almeida-Filho, E. S. (2011). *Listeria spp.* e *Listeria monocytogenes* na produção de salsichas tipo hot dog. Ciência Animal Brasileira 12: 339-352.
- Campos, L. C. & Silva, D. C. V. (2008). Listeria Monocytogenes. In: L.R. Trabulsi, F. Althertum, M.L.B.O. Racz, Microbiologia (pp. 237). São Paulo: Editora Atheneu.
- Barmpalia, I. M., Koutsoumanis, K. P., Geornaras, I., Belk, K. E., Scanga, J. A., Kendall, P. A., Smith, G .C. & Sofos, J. N. (2005). Effect of antimicrobials as ingredients of pork bologna for *L. monocytogenes* control during storage at 4 or 10°C. Food Microbiology 22: 205–211.
- Couture, H. (2009). *L. monocytogenes*. Evaluation Division, Bureau of Microbial Hazards, Health Canada. Access at: http://www.cmc-cvc.com/english/documents/ CMCListeriapolicyoctober12009HeleneCouture.pdf
- Nørrung, B., Andersen, J. K. & Buncic, S. (2009). Main concerns of pathogenic microorganisms in meat. In: F. Toldrá, Safety of Meat and Processed Meat (pp. 3-29). New York: Springerlink.
- Sofos, J. N. & Geornaras, I. (2010). Overview of current hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157:H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. Meat Science 86: 2-14.
- FDA, 2003. *L. monocytogenes* Risk Assessment: I. Introduction. FDA/Center for Food Safety and Applied Nutrition, USDA/Food Safety and Inspection Service. Access at: http://www.fda.gov/Food/ScienceResearch/ResearchAreas/RiskAssessmentSafetyAssessment/ucm

184052.htm.

- EC, 2005. Regulation (EC) Nº 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs (Official Journal of the European Union L 338/1).
- HC, 2011. Health Canada, policy on *Listeria monocytogenes* in ready-to eat foods. Access at: http//www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php.
- Greenberg, J. & Elliott, C. (2009). A cold cut crisis: listeriosis, Maple Leaf Foods, and the politics of apology. Canadian Journal of Communication 34: 190-203.
- Burt, S. A. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94: 223-253.
- Schuenzel, K. M. & Harrison, M. A. (2002). Microbial antagonists of foodborne pathogens on fresh minimally processed vegetables. Journal of Food Protection 65: 1909-1915.
- Skandamis, P. N. & Nychas, G. E. (2002). Preservation of fresh meat with active and modified atmosphere packaging conditions. International Journal of Food Microbiology 79: 35-45.
- Burt, S. A. & Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology 36: 162-167.
- Klancnik, A., Piskernik, S., Jersek, B. & Mozina, S. S. (2010). Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. Journal of Microbiological Methods 81: 121-126.
- Apostolidis, E., Kwon, Y. I. & Shetty, K. (2007). Inhibition of *Listeria monocytogenes* by oregano, cranberry and sodium lactate combination in broth and cooked ground beef systems ans likely mode of action through proline metabolism. International Journal of Food Microbiology 128: 317-324.
- Wen, A., Delaquis, P., Stanich, K. & Toivonen, P. (2003). Antilisterial activity of selected phenolic acids. Food Microbiology 20: 305-311.