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ORIGINAL ARTICLE

Antilisterial effects of antibacterial formulations containing essential oils, nisin, nitrite and organic acid salts in a sausage model

Mina Ghabraie[1] · **Khanh Dang Vu**[1] · **Tanzina Huq**[1] · **Avik Khan**[1] · **Monique Lacroix**[1]

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Abstract This study was conducted to evaluate the effects of sixteen antibacterial formulations against *Listeria monocytogenes* in a sausage model using a standard experimental design with 4 independent factors at 2 levels (2^4). Four independent factors consisted of nisin (12.5–25 ppm), nitrite (100–200 ppm) and organic acid salts (1.55–3.1 %) and the mixture of Chinese cinnamon and Cinnamon bark Essential Oils (EOs) (0.025–0.05 %). Based on the analysis, utilization of low (0.025 %) or high concentration (0.05 %) of EOs in combination with low concentration of nitrite (100 ppm), organic acid salts (1.55 %), and nisin (12.5 ppm) could reduce respectively 1.5 or 2.6 log CFU/g of *L. monocytogenes* in sausage at day 7 of storage as compared to the control. A predictive equation was created to predict the growth of *L. monocytogenes* in sausage. The sensory evaluation was then performed on selected optimized formulations in cooked meat (both pork and beef sausages) with a trained jury consisting of 35 individuals, demonstrated the selected antimicrobial formulations were organoleptically acceptable. The results revealed an important role of hurdle technology to control *L. monocytogenes* in meat product.

Keywords *Listeria monocytogenes* · Essential oil · Hurdle technology · Sausage · Organoleptic properties

✉ Monique Lacroix
monique.Lacroix@iaf.inrs.ca

[1] Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center, INRS-Institut Armand-Frappier, Institute of Nutraceutical and Functional Foods, 531, Boulevard des Prairies, Laval, Québec H7V 1B7, Canada

Introduction

Meat and meat products, due to their high levels of bacterial nutrients, pH (5.5–7.0) and high water activity, offer a congenial environment for the growth of bacteria (Dave and Ghaly 2011). Among foodborne pathogens, *L. monocytogenes* is one of the most dangerous ones. It is a Gram-positive bacterium, facultative anaerobe pathogen and causes listeriosis which is a severe human disease associated with meningitis, gastroenteritis, etc. (Solomakos et al. 2008b). It was reported that *L. monocytogenes* causes 19 % foodborne illness in USA annually (Scallan et al. 2011). Meat products can be contaminated by *L. monocytogenes* during preparation, storage and distribution (Fratianni et al. 2010). The contamination of *L. monocytogenes* can also be happened due to inadequate temperature for cooking or post contamination with contaminated hand, knife or other dishes (Ramaswamy et al. 2007; Samaxa et al. 2012).

Nowadays, consumers concern about probable toxic and carcinogenic effects of synthetic antimicrobial agents used in food products (Jayasena and Jo 2013). Essential Oils (EOs) are among of the natural compounds that can be used in food products as they are generally recognized as safe (GRAS) (Oussalah et al. 2007) and their antimicrobial efficacy against several foodborne pathogens, especially *L. monocytogenes* (Burt 2004; Fratianni et al. 2010). Some EOs such as oregano, rosemary, thyme and clove have shown their potential as antimicrobial agents that can be used in meat and meat products (Jayasena and Jo 2013).

In order to avoid possible side effects of EOs and to enhance their antimicrobial efficacy (probable synergetic effect), EOs are widely using in combination with other antimicrobial agents (Solomakos et al. 2008a, b). For instance nisin can be used as it is a candidate antimicrobial compound against *L. monocytogenes* for meat application (Abdollahzadeh et al.

2014; Millette et al. 2007; Solomakos et al. 2008b). Nisin is a ribosomally synthesized cationic polypeptide having antimicrobial activity. It is a heat-stable compound produced by *Lactococuss lactis* (Millette et al. 2007). It is approved and widely used in more than 50 countries (Abdollahzadeh et al. 2014). Potassium lactate (PL) and sodium acetate (SA) are also widely used as food preservatives as they are recognized as safe (GRAS) (Perumalla et al. 2012). Nitrate (NO_3^-) has been used since 19th century as a food preservative, especially for meat and meat products. The reduction of nitrate to nitrite (NO_2^-) by microorganisms present in the meat causing the meat red color by producing NO-myoglobin (Honikel 2008). USDA/FSIS regulates ≤200 ppm for $NaNO_2$ and ≤500 ppm for $NaNO_3$ in meat products (Nyachuba et al. 2007). However, it would be better to use lower concentration of nitrate or nitrite (Cammack et al. 1999).

Combined treatment or hurdle technology is of interest for food preservation. It helps to prevent the bacterial resistance to individual antibacterial treatment. It also helps to reduce the dosage of each treatment since the combined treatment may cause synergistic antibacterial effects between or among antibacterial agents/treatments against target bacteria (Zhou et al. 2010).

Further, in order to improve the activity of the antimicrobial compounds during storage, microencapsulation in edible polymers has been found as an effective technology. Microencapsulation could also decrease the organoleptically affection of antimicrobial agents, causes the slow rate release of EOs during storage time, protecting antimicrobial agents to be impaired by contacting the food matrix, promote the efficiency of them and so on (Hyldgaard et al. 2012). Hydrocolloids such as proteins, cellulose derivatives, alginates, pectins, starches, and other polysaccharides are basic component of edible coating or polymeric matrix for encapsulating antimicrobial agents (Neetoo et al. 2010).

The objective of this study was to develop antilisterial formulations containing essential oils, nisin, nitrite and organic acid salts in meat model (fresh pork sausage) by using a standard full factorial design. These antilisterial formulations were encapsulated in Ca-alginate to improve their effects during storage. Sensorial evaluation was carried out in order to verify the organoleptic acceptance of the optimized antimicrobial formulation.

Materials and methods

Materials

Tryptic soy broth (TSB), Peptone and Palcam agar were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). Alginate and $CaCl_2$ were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Cellulose Nano Crystal (CNC) was supplied from FPInnovations pilot plant (Pointe-Claire, QC, Canada). Nisin (Niprosin™, purity 2.5 %, 77.5 % salt and 20 % vegetable protein, Profood, Naperville, IL, USA) was purchased from Pro-food International Inc. Ground lean pork meat was purchased from a local grocery store (IGA, Laval, Quebec, Canada). Binding agent and sodium erythorbate were delivered from BSA Food Ingredients (St-Leonard, Quebec, Canada). Chinese cinnamon EO (containing mainly *Trans*-Cinnamaldehyde (87.58 %) and cinnamyl acetate (7.53 %)) and Cinnamon bark EO (containning mainly *Trans*-cinnamaldehyde (40.71 %), cinnamyl acetate (14.25 %), β-phellandrene (9.02 %), and β-caryophyllene (7.41 %)) were provided by Aliksir Inc. (Grondines, Québec, Canada). EOs were mixed together at a ratio of 1: 4 based on our previous study.

Bacterial strain

L. monocytogenes (HPB 2812) was stored at −80 °C in Tryptic Soy Broth (TSB) medium (TSB; BD, Franklin Lakes, NJ, USA) containing glycerol (10 % *v*/v). Before each experiment the bacteria were propagated through two consecutive 24 h in 9 ml of TSB at 37 °C. The final concentration of bacteria after two times of propagation was approximately 10^9 CFU/ml. The culture was used as working culture for inoculation into sausage.

Experimental design for antimicrobial formulations

The preliminary study was done to examine the antimicrobial activity of different organic acid salts as in meat. Based on the results, it was found that the mixture of 0.40 % (*w*/w) sodium acetate and 2.70 % (*w*/w) potassium lactate caused a bacterial reduction by less than 0.5 log CFU/g at day 7 as compared to the control. Therefore they were chosen to be used in this study. In our previous results, mixed EOs of Chinese cinnamon and Cinnamon bark (0.05 %, *v*/*w*) could reduce the growth of *L. monocytogenes* by less than 0.5 log CFU/g meat during 7 days of storage at 4 °C. In case of nisin, it was found that 1000 IU/g minced beef (25 ppm) can reduce around 1 log CFU/g minced fish during storage of 12 days (Abdollahzadeh et al. 2014). Thus, in the current study, we selected to use 25 ppm nisin, 200 ppm nitrite, 0.05 %, *v*/*w* mixed EOs, and 3.1 %, *w*/w mixed organic acid salts (potassium lactate plus sodium acetate) at high concentrations (high level) in the experimental design. The lower concentrations (low level) of nitrite, nisin, mixed EOs and mixed organic acid salts in the experimental design were 100 ppm, 12.5 ppm, 0.025 %, *v*/*w* and 1.55 %, *w*/w, respectively. A standard experiment with 4 independent factors at 2 levels (2^4) was conducted using STATISCA 8 (STATSOFT Inc., Thulsa, US). There were 16 antibacterial formulations (experimental runs) in the design (Table 1). The dependent factor was the count (log CFU/g meat) of *L. monocytogenes* at day 7.

Table 1 Final concentration of *Listeria monocytogenes* (log CFU/g meat) in sausage samples at day 7 stored at 4 °C

Formulations	Nitrite (ppm)	Nisin (ppm)	Organic acid salts (%, *w*/w)	Essential oil (%, *v*/*w*)	Log CFU/g meat
1	100	12.5	1.55	0.025	2.6 ± 0.2
2	200	12.5	1.55	0.025	3.1 ± 0.1
3	100	25	1.55	0.025	2.6 ± 0.2
4	200	25	1.55	0.025	2.4 ± 0.1
5	100	12.5	3.1	0.025	2.9 ± 0.3
6	200	12.5	3.1	0.025	2.4 ± 0.3
7	100	25	3.1	0.025	1.8 ± 0.3
8	200	25	3.1	0.025	1.5 ± 0.0
9	100	12.5	1.55	0.05	1.8 ± 0.2
10	200	12.5	1.55	0.05	2.1 ± 0.3
11	100	25	1.55	0.05	1.6 ± 0.2
12	200	25	1.55	0.05	2.1 ± 0.3
13	100	12.5	3.1	0.05	1.9 ± 0.3
14	200	12.5	3.1	0.05	2.2 ± 0.3
15	100	25	3.1	0.05	1.6 ± 0.2
16	200	25	3.1	0.05	1.7 ± 0.2
Control	0	0	0	0	4.3 ± 0.2

Nisin preparation

Nisin (0.25 *w*/*v*) was prepared by mixing Niprosin™ powder in 100 mL 0.01 M $CaCl_2$ solution and the pH of the nisin-$CaCl_2$ solution was adjusted to around 3 by diluted lactic acid. The nisin-$CaCl_2$ solution was centrifuged for 15 min at 3500×g at 4 °C to remove the undissolved particles and collected the nisin-$CaCl_2$ supernatant (Huq et al. 2015).

Microencapsulation of antimicrobial formulations

All the antimicrobial factors were microencapsulated into alginate–CNC (Cellulose Nanocrystal) microbeads followed by (Huq et al. 2015) before adding to the food model. Alginate is an efficient carrier for various antimicrobial treatment in food due to its probable interaction with incorporated antimicrobials and it has not detectable taste (Neetoo et al. 2010). The microbeads suspension was prepared by mixing 2 % (*w*/*v*) of alginate (guluronic acid ~65–70 %; mannuronic acid content ~5–35 %) in deionized water under magnetic stirring. A 1 % (*w*/*v*) CNC suspension was prepared by dispersing spray dried CNC powder in deionized water under magnetic stirring. Then, the CNC suspension was subjected to ultra-sonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g of CNC. A 5 % (*w*/w) CNC from 1 % CNC suspension (according to weight percentage of alginate) and 2.5 % (*w*/w) of Tween 80 (emulsifier) were mixed with 2 % (*w*/*v*) alginate suspension. All 16 different formulations containing nitrite, nisin, organic acid salts and mixed EOs were prepared separately. The proportion amount of each antimicrobial formulation was added to alginate-CNC suspension and homogenized by Ultra-Turrax TP18/1059 homogenizer (Janke & Kunkel, Staufen, Germany) at 25,000 rpm for 3 min. All the calculation was done in terms of sausage weight (20 g).

Preparation of sausage with different microencapsulated antimicrobials

The sausage was prepared by mixing lean ground pork (70 % *w*/w), binding agent (8 % *w*/w) and water (22 % *w*/w). Sodium erythorbate (750 ppm) was mixed with sausage according to total meat weight. Then each 20 g of sausage was put in a bag and kept at −80 °C under vacuumed packaging. For sterilization, the sausages were irradiated at 45 kGy before applying the antimicrobial formulation and inoculating with bacteria.

A 4 ml of emulsified microbeads were applied on each 20 g of sausage and mixed by Lab-blender 400 Stomacher (Laboratory Equipment, London, UK) for 2 min at 230 rpm. Then the sausage samples were inoculated with diluted working culture of *L. monocytogenes* to have a final concentration of approximately 10^3 CFU/g and mixed for another 2 min at 230 rpm. Finally, the samples were packed under vacuum and stored at 4 °C for storage. Control sausage samples containing 4 ml of microbeads without antibacterial agents were also included. Since it is considered the meat model as fresh sausage in which the shelf life is normally less than 7 days (Savic 1985). Thus, microbial analysis was conducted at day 7 of storage.

Microbiological analysis

Each sample was transferred to a stomacher bag and diluted with peptone water (0.1 % *w/v*). The sample was homogenized in a Lab-blender 400 Stomacher for 1 min at 230 rpm. From each homogenate sample, serial decimal dilutions were done in peptone water (0.1 % *w/v*). Then 100 µl of each dilution was spreaded on Palcam agar plate. Palcam agar was prepared by the addition with antibiotics acriflavine (5 mg/l), polymyxin B (10 mg/l) and ceftazidime (8 mg/l) in order to get the selective enumeration of *L. monocytogenes*. In parallel with the experimental design, a control sausage containing encapsulation matrix (alginate-Ca) without antimicrobial agents was also conducted. After 48 h incubation at 37 °C, bacterial colonies were counted and expresses as log CFU/g of sausage.

Sensory evaluation

The sensorial analysis was performed to verify if the selected formulations would change the organoleptic properties of meat or not. The treated sausages were prepared in the same way as microbial analysis with 2 different antimicrobial formulations (without inoculation of *L. monocytogenes*). The sausages were cooked at 400 ° F (~200 °C) for 10–15 min in an oven and 15 g of each sample was served to panelists in warm conditions. Each sample was coded with 3-digit random number. The jury team consisted of 35 examiners who were trained for evaluating organoleptic properties of food (Département Techniques de diététique et Gestion d'un établissement de restauration, Collège Montmorency). The panelists scored the sensory odor, texture and taste of samples by using 9-point hedonic scale (9 = Like extremely, 8 = Like very much, 7 = Like moderately, 6 = Like slightly, 5 = Neither like nor dislike, 4 = Dislike slightly, 3 = Dislike moderately, 2 = Dislike very much, 1 = Dislike extremely) (Poste et al. 1991). According to the scale, the values more than 5 were considered organoleptically acceptable. The jury team had unsalted biscuit and glass of water to eat and drink between each samples.

Statistical analysis

All the experiment were conducted in duplicate. For each replicate, three samples were analyzed. The obtained data of the count (concentration) of *L. monocytogenes* (log CFU/g) in 16 formulations of the experimental design were used for analysis of variance (ANOVA) and regression analysis using software STATISTICA 8. An equation (or a model) consisted of linear effect of each independent factor and interactive effects among independent factors were built to predict the concentration of *L. monocytogenes* in sausage consisting different concentrations of antibacterial agents (Eq. 1).

$$Y = A_0 + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{4} A_{ij} X_i X_j \quad (1)$$

Where *Y*, predicted response (concentration of *L. monocytogenes*, log CFU/g sausage); A_o, constant coefficient; X_i and X_j, values of various levels of the independent variables; *Ai*, values of linear coefficients; *Aij*, interactive coefficient between two independent factors.

For sensorial analysis, the results were expressed as mean ± SD. One-way analysis of variance (ANOVA) tests using SPSS program (IBM Corporation, Somers, NY, USA) was conducted to analyze the data of sensorial analysis results (Poste et al. 1991). Duncan's multiple range tests was used to compare the mean values. Difference between mean values at $P \leq 0.05$ was considered significantly.

Results

The concentrations of *L. monocytogenes* of 16 antimicrobial formulations at day 7 of storage are presented in Table 1.

Regression analysis of the experimental design

ANOVA analysis performed on the data (concentration of *L. monocytogenes*, log CFU/g) obtained at day 7 showed the regression coefficient (R^2) of the model was 0.95. The R^2 is the percent of the response variation explained by the model and represents how well the model fits with the data. Regression analysis were also carried out in order to determine the significance of the linear, and interactive coefficients of independent factors on the growth of *L. monocytogenes* and build a predictive equation (a model). Table 2 represents the regression coefficients of linear and interactive effects of 4 independent factors (nitrite, nisin, organic acid salts (OAS) and EOs) of the model. The linear effect of nitrite, nisin and OAS are not important in the model since the *P* values of these factors are higher than 0.3 whereas EOs showed the linear negative effect with $P < 0.002$. This means that EOs mixture is the most important factor in the model in which EOs could reduce the growth of *L. monocytogenes*. It can be observed that there are significant interactive effects among 4 independent factors on the growth of *L. monocytogenes* in the sausage. The interactive effects of nitrite x nisin ($P \leq 0.1$) or nisin x OAS ($P \leq 0.05$) caused a decrease in the growth of *L. monocytogenes*. The interactive effects between nitrite and EOs, or nisin and EOs or OAS and EOs are positive interactive effects at $P \leq 0.1$ (Table 2).

Table 2 Regression coefficient of linear and interactive effects of 4 independent factors of the equation

Factors	Regression coefficient	*P* value
Intercept	5.02	0.0018
Linear effects		
Nitrite (X_1)	0.00	0.9733
Nisin (X_2)	−0.03	0.3899
Organic acid salts (OAS) (X_3)	0.16	0.6215
Essential Oil (X_4)	−101.46	0.0017
Interactive effects		
X_1X_3	−0.002	0.0886
X_1X_4	0.168	0.0581
X_2X_3	−0.02	0.0399
X_2X_4	1.33	0.0604
X_3X_4	12.51	0.037

Generally, the regression coefficients of linear or interactive effects will be included in the equation when their *P* values are less than or equal 0.05 ($P \leq 0.05$), however, in some cases, it is necessary to consider other factors even their *P* values are smaller or equal to 0.1 ($P \leq 0.1$) to ensure a good fit equation for the prediction. In this case, it is found that all factors with *P* values ≤ 0.1 are necessary to include into the equation to predict the concentration of *L. monocytogenes* in the sausage. The final model is presented in the following Eq.:

$$Y = 5.023 - 101.45X_4 - 0.02X_1X_3 + 0.168X_1X_4 - 0.024X_2X_3 + 1.33X_2X_4 + 12.51X_3X_4$$

Where:

Y is the dependent factor of the model (the concentration of *L. monocytogenes*, log CFU/g in sausage meat product)

X_1 is the concentration of nitrite (ppm)
X_2 is the concentration of nisin (ppm)
X_3 is the concentration of OAS (%, *w*/*w*)
X_4 is the concentration of essential oil (%, *v*/*w*)

Response surface plots

The concentration of *L. monocytogenes* in the control sausage without antibacterial agents at day 7 was 4.3 ± 0.2 (Table 1). To see the interaction effects among independent factors on the growth of *L. monocytogenes*, response surface plots were created and presented in Figs. 1 and 2.

Figure 1 presents the antibacterial effect of nisin and OAS on the growth of *L. monocytogenes* when nitrite and EOs are fixed at low concentration of 100 ppm and 0.025 %, respectively. It can be observed that at this condition, high nisin concentration (24–26 ppm) and high OAS concentration (2.8–3.2 %, *w*/w) could cause the growth of *L. monocytogenes* less than 1.8 log CFU/g sausage which is less than that of control sausage (4.3 log CFU/g) by more than 2.5 log CFU, or when nisin concentrations are from 20 to22 ppm and OAS concentrations are from 2.4 to 3.2 %, the concentration of *L. monocytogenes* is less than 2.2 log CFU/g sausage which is less than that of control sausage by more than 2 log. These results are interesting, however, in these cases required high nisin and OAS concentrations. When nisin concentrations slightly change from 14 to 16 ppm and combining with OAS from low to high concentration (1.2–3.2 %), the concentration of *L. monocytogenes* in sausage becomes less than 2.6 log CFU/g. This bacterial count is significant lower than that of control sausage (4.3 log CFU/g) by around 1.7 log.

Figure 2 presents the antibacterial effects of nitrite and EOs on the growth of *L. monocytogenes* when nisin and OAS are fixed at low concentration of 12.5 ppm and 1.55 %, respectively. In this condition, it can be observed that when EOs concentrations are from 0.045 to 0.05 % (*v*/*w*) and with low nitrite concentrations from 100 to 160 ppm, the concentration of *L. monocytogenes* in sausage is less than 1.7 log CFU/g, which is less than that of control sausage by more than 2.6 log CFU/g. The Fig. 2 also showed that utilization of high nitrite concentration (more than 160 ppm) in the formulations that contains high concentration of EOs (0.05 %) and low concentrations of nisin and OAS might cause an increase in the growth of *L. monocytogenes* by more than 1.8 log CFU/g. Thus, the formulation containing EOs (0.05 %, *v*/*w*), low concentrations of nitrite (100 ppm), nisin (12.5 ppm) and OAS (1.55 %, *w*/w) can be considered as the best formulation in decreasing the growth of *L. monocytogenes* (Fig. 2). This formulation (Formulation A) was then chosen for sensorial evaluation in meat products.

It is also of interest to find that EOs at low concentration of 0.025 % and in combination with low concentrations of nitrite (100 ppm), nisin (12.5 ppm) and OAS (1.55 %, *w*/w) could cause the growth of *L. monocytogenes* by approximately 2.8 log CFU/g, which is still less than that of control by 1.5 log CFU/g (Fig. 2). This formulation (Formulation B) was also chosen for sensorial evaluation in meat products to compare with the best formulation above.

Sensorial properties of selected antilisterial formulation in meat products

The results of sensorial analysis are presented in Table 3. Results showed both of the selected formulations were acceptable (more than 5) in term of texture, smell and taste in both fresh beef sausage and fresh pork sausage as compared to those of control without antibacterial formulations.

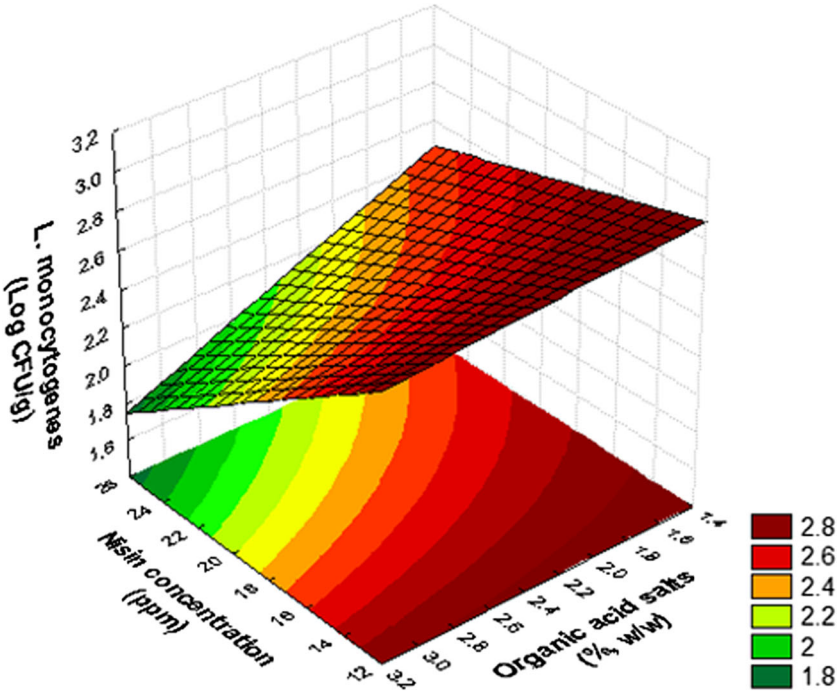

Fig. 1 Effect of nisin and organic acids salt on the growth of *L. monocytogenes* (log CFU/g meat) (nitrite and EOs are fixed at low levels, 100 ppm and 0.025 %, respectively)

Discussion

The experimental design was done to find the most effective antimicrobial formulation against *L. monocytogenes* in fresh pork sausage. All the formulations were able to decrease at least 1 log of bacteria as compare to the control sausage (4.3 log CFU/g) after 7 days of storage (Table 1). The antimicrobial formulation with lower concentrations of nitrite, nisin, organic acid salts and EOs were also effective against *L. monocytogenes* which approved that we can reduce the acceptable concentration of each tested antimicrobial agent to half and still have antimicrobial safety (more than 1.5 log reduction as compared to the control).

Chinese cinnamon and Cinnamon bark at the organoleptic acceptable concentration (0.05 %) was used in this study as the highest concentration. Combination of different processes can have synergistic or additive antimicrobial effects and therefore, ensure microbial safety (Jayasena and Jo 2013). In

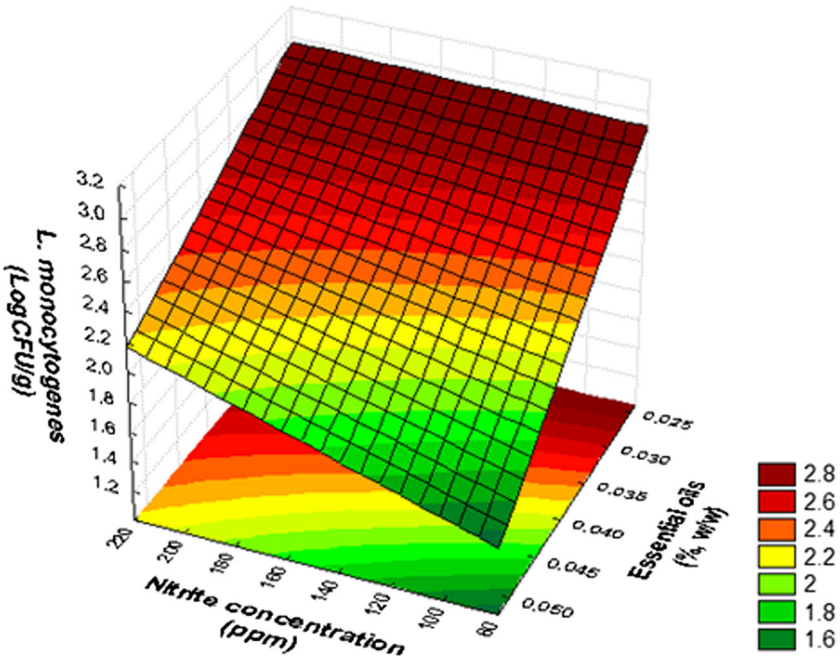

Fig. 2 Effect of nitrite and EOs on the growth of *L. monocytogenes* (log CFU/g meat) (nisin and organic acid salt are fixed at low levels, 12.5 ppm and 1.55 %, respectively)

Table 3 Sensorial evaluation of two kinds of fresh sausage with two selected antimicrobial formulations

	Texture		Smell		Taste	
	Pork	Beef	Pork	Beef	Pork	Beef
FA[1]	6.08 ± 1.88[a]	6.22 ± 1.64[a]	6.50 ± 1.88[a]	5.69 ± 1.80[a]	5.83 ± 2.43[a]	4.86 ± 2.03[a]
FB	6.44 ± 2.02[a2]	6.41 ± 1.40[a]	6.61 ± 1.51[a]	5.58 ± 1.66[a]	5.86 ± 2.11[a]	5.19 ± 2.26[a]
FC	6.25 ± 1.55[a]	6.44 ± 1.59[a]	5.91 ± 1.99[a]	5.91 ± 1.96[a]	5.16 ± 1.91[a]	5.05 ± 2.30[a]

[1] FA is the formulation A, containing low concentrations nitrite (100 ppm), nisin (12.5 ppm), organic acid salts (1.55 %), and high concentration of EOs (0.05 %, *v/w*). FB is the formulation B, containing low concentrations of EOs (0.025 %, *v/w*), nitrite (100 ppm), nisin (12.5 ppm) and organic acids salt (1.55 %). FC is the control without antimicrobial agents

[2] The same lower case letters within each columns indicate the values are not significantly different ($p > 0.05$)

current study, cinnamaldehyde is the major component of selected EOs. Chinese cinnamon and Cinnamon bark has 87.58 % and 40.71 % of trans-cinnamaldehyde, respectively. Aldehyde (CHO-) could covalently cross-link with bacterial DNA and proteins. According to literature, cinnamaldehyde at higher concentration can inhibit the ATPase and at lethal concentration, it disturbs the cell membrane (Hyldgaard et al. 2012). At high but sub-lethal concentration, cinnamaldehyde can access to periplasm and decrease the activity of transmembrane ATPase. According to Burt (2004) and Hyldgaard et al. (2012), EOs can eliminate the bacteria by damaging the cell membrane, inhibit some of the enzymes such as those involved in the synthesis of ATP, periplasmic enzyme, amylase, protease and so forth, and produce covalent cross-link with DNA. Increasing the permeability and depolarization of membrane are two main ways which antimicrobial compounds act on the membrane. The results also revealed that EOs is the most important factor in reducing the growth of *L. monocytogenes* which is similar with regression analysis of the model.

Edible polymer was used for encapsulation of antibacterial agents in this study since it has been demonstrated that encapsulated antimicrobial agents has higher inhibitory activity than antimicrobial agents without encapsulating (Huq et al. 2014). In addition, entrapping reduces the probable negative organoleptic effect of each of components on food. Furthermore encapsulated EOs in edible polymer causes a slow rate release of EOs, so their antibacterial activity would last for long time whereas in dipping or spraying EOs the diffusion would be continued into the food and microorganism might grow on the surface (Neetoo et al. 2010).

Nisin cause bacterial cell death by binding to the peptidoglycan layer and causing destabilization of cytoplasmic membrane with forming pores (Solomakos et al. 2008a). It has been approved that nisin has antilisterial activity in meat system. The effect of nisin could be weakened due to its binding with proteins or fat in meat products (Solomakos et al. 2008b). The efficiency of nisin can be affected by pH, food processing and food ingredients, too (Abdollahzadeh et al. 2014). So encapsulation of nisin in edible polymer could protect this antimicrobial agent from food ingredients. When nisin is used in combination with other antimicrobial agents like EOs, additive antibacterial effect could be observed because EOs could disintegrate the protective outer membrane which make the bacteria more sensitive to nisin (Solomakos et al. 2008a).

Bacteriocin alone cannot ensure the safety, they have to be combined with other technologies or other antimicrobial agents such as sodium acetate or potassium lactate (Zacharof and Lovitt 2012). Apostolidis et al. (2008) have confirmed the increase of antimicrobial activity of combined EOs with salt of organic acids such as potassium lactate against *L. monocytogenes*. In fact lactates can inhibit the growth of bacteria by reducing the water activity of food products follows by retarded development of bacteria and also by acidifying the intracellular pH (Stekelenburg 2003). Potassium lactate is derived from lactic acid and it can extend the lag phase of pathogenic bacteria thereby prolong the shelf life of food (Stekelenburg 2003). Several studies demonstrated the antimicrobial activity of sodium acetate in different food system. For instance, Manju et al. 2007 used 2 % of sodium acetate and combined it with vacuum-packaging and extends the shelf life of seafood to 15 days. Blom et al. 1997 demonstrated that sodium acetate individually at the concentration of 0.5 % could inhibit the growth of *L. monocytogenes*. In our recent study, other OAS such as sodium acetate, potassium lactate and calcium propionate were also found as important antibacterial agents to reduce the growth rate of *L. monocytogenes* in ham (Dussault et al. 2016).

The $NaNO_2$ or KNO_2 are agents for curing process (Honikel 2008). Nitrite is a very reactive substance and produces several reactions in meat and that is why the concentration of that should be controlled. The sum of both nitrite and nitrate is critical for human body because nitrate can be reduced to nitrite in oral cavity and in stomach, due to acidic environment and nitrite can form carcinogenic nitrosamines (Honikel 2008). According to Honikel (2008), the antibacterial mechanism of nitrite is not understood yet, while literature revealed that nitrite slows or control the growth of *L. monocytogenes* but not stop the growth of this bacterium (Myers et al. 2013).

The synergetic effect of $NaNO_2$ and EOs is important for food companies as it caused to use both of them at lower concentration. Lower nitrite is preferable for consumers and lower EO is promising for not influencing (affecting) the sensorial properties of food products, especially meats (Cui et al. 2010). In this study, it was found that high nitrite concentration (more than 160 ppm) may not be necessary to be used in combination with high EOs concentration to inhibit the growth of *L. monocytogenes* in sausage meat (Fig. 2) and therefore, low nitrite (100 ppm) can be applied together with other antibacterial agents to ensure the food safety of this product.

Conclusion

To promote the safety of meat products, combination of mild preservation technologies is important. Our results demonstrated that combination of different antimicrobial agents and encapsulation in microbeads alginate could reduce the growth of *L. monocytogenes* in fresh pork sausages significantly as compared to that of control. The formulation A (mixed EOs (0.05 %, *v*/*w*), mixed organic acid salts (1.55 %, *w*/w), nisin (12.5 ppm) and nitrite (100 ppm)) and formulation B (mixed EOs (0.025 %, *v*/*w*), mixed organic acid salts (1.55 %, *w*/w), nisin (12.5 ppm) and nitrite (100 ppm)) caused the reduction of *L. monocytogenes* by more than 2.6 and 1.5 log CFU/g sausage as compared to that of control. The two formulations were also organoleptically accepted in both pork and beef sausages.

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