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Original Article

Determination of the Minimum Inhibitory Concentration of the Barberry Extract and the Dried Residue of Red Grape and Their Effects on the Growth Inhibition of Sausage Bacteria by Using Response Surface Methodology (RSM)

Fatemeh Riazi[1*], Fariba Zeynali[2], Ebrahim Hosseni[3], Homa Behmadi[4]

1- M.Sc Student, Dept.of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, Iran

2- Assistant Professor, Dept.of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, Iran

3- Associate Professor, Collage of Food Science and Technology, Faculty of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

4- Member of Scientific Board, Agricultural Engineering Research Institute, Food Engineering and Post-Harvest Technology. Res. Dept, Tehran, Iran

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ABSTRACT

Background and Objectives: With regard to the hazards of nitrite, application of natural preservatives in order to reduce the microbial load of meat and meat products is increasing. Owing to their anti-bacterial properties, red barberry and the dried residue of red grape could be suitable replacers for nitrite.

Materials and Methods: Agar dilution method was employed in order to determine the minimum inhibitory concentration (MIC) of the barberry extract and the dried residue of red grape. The anti-microbial effects of the barberry extract (0-600 mg/kg), the dried residue of red grape (0-2%) and nitrite (30-90 mg/kg) were investigated on the total viable counts of *Clostridium perfringens*, as well as on the psychrophilic bacteria after 30 days of storage at 4°C. Finally, the effects of the three independent variables in the optimal sample were examined on the growth of the inoculated *C. perfringens*.

Results: The MIC of the barberry extract and the dried residue of red grape on *Staphylococcus aureus* was 3 and 6 (mg/ml), respectively. In the case of *Escherichia coli*, it was 4 and 7 (mg/ml), respectively. The barberry extract and nitrite reduced the growth of the living aerobic bacteria significantly. The spores of the inoculated *C. perfringens* had no growth in the optimum sample during storage.

Conclusions: The barberry extract and the dried residue of red grape as natural preservatives, could partially substitute for nitrite in order to reduce the microbial load of sausage.

Keywords: Barberry extract, Dried residue of red grape, Nitrite, Minimum inhibitory concentration, Sausage

Introduction

Nowadays, the demand for the ready-to-eat meat products is increasing because of the rise in people`s businesses [1]. Sausage is one of the most important and popular meat products throughout the world. The production process of this product is composed of mixing the meat with the curing, flavoring and coloring agents to obtain a product of acceptable sensory and technological properties [2]. Meat products might be contaminated by the microorganisms transmitted by meat handlers, persons who transmit pathogenic microorganisms during the production process, packaging, and marketing.

C. perfringens is a spore-forming Gram-positive obligate anaerobic Basil, which is spread out in nature (soil, water, air and food) in addition to the digestive systems of humans and animals. This bacterium produces more than 13 different types of toxins, and is usually categorized into five classes (A, B, C, D and E) on which the production of four principle deadly toxins (α, β, ε and iota) depends [3]. It is able to grow in the temperature range of 15-50°C and at

***Address for correspondence:** Fatemeh Riazi, M.Sc Student, Dept.of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, Iran. E-mail address: riazi_fa@yahoo.com

relatively higher temperatures, especially at 43-46°C [4] with the generation time of 7-8 min in peptone water or thioglycolate [5]. *C. perfringens* has been reported as one of the most prevalent bacterial agents for foodborne diseases in the USA. Enterotoxin is the most important factor of the foodborne disease of this bacterium. It is released in the intestine along with the endospores, which cause severe ache and abdominal cramps in the host [6].

Application of curing agents (mainly nitrite) is one of the most effective actions to control microbial spoilage, especially *Clostridium botulinum* in sausage. However, in addition to its function as an antioxidant, it could have a considerable impact on color, odor and flavor of sausage [7]. During cooking, nitrite can combine with the amines present in meat and produce the carcinogenic compounds of n-nitrosamine [8]. In response to the current consumers` demands for healthier and safer products whose sensory attributes (color, odor, flavor, etc.) have been preserved, researchers have been trying for the reduction or removal of the existing nitrite from meat products with the least possible changes. In this regard, various substances have been utilized as substitutes for the added nitrite to assure that these additives play their roles in the final product while retaining its quality [9-11].

Berberis vulgaris, a member of the *Berberidaceae* family, is used as a medicinal plant to remedy fever, edema, sore throat, diarrhea, parasitic infections, and Trichoderma infection. During the recent years, various species of barberry, including *B. vulgaris*, have been investigated well for their potential health-promoting effects such as anti-microbial, anti-inflammatory and anti-cancer properties [12]. Barberry possesses many alkaloids among which berberine (an iso-conjugated alkaloid) has anti-fungal and anti-bacterial effects [13]. In a study carried out by Freile et al. (2003) on the anti-microbial effect of the aqueous extract and purified berberine isolated from *B. heterophylla*, it was observed that purified berberine had anti-bacterial effect on Staphylococcus aureus and anti-fungal effect on *Candida* [14].

Grape is one of the most ancient fruits cultivated all over the world. Grape residue is one of the most important products of grape processing, which is widely produced throughout the world annually. It is either burned or utilized as animal feed [15]. Grape residue is a rich source of polyphenols, which in turn have anti-microbial effects [16]. So far, no research has been reported for the anti-microbial effect of the dried residue of red grape. At the same time, grape seed has anti-microbial effect on the pathogenic agents of foodstuffs. Su cha et al. (2002) evaluated the anti-microbial effect of grape seed extract on *Listeria innocua, Escherichia coli, Salmonella enteritidis, Staphylococcus aureus* and *Micrococcus luteus* through the agar diffusion method, and came to conclusion that the grape seed extract prevented the growth of all the above-mentioned bacteria [17].

The objective of this study was to study the anti-microbial effects of the barberry extract and the dried residue of red grape and their impacts on the microbial properties of sausage.

Materials and Methods

Preparation of the barberry extract: Red barberry (*Berberis Vulgaris Var.Asperma*) was supplied from the city of Qaen and dried until its moisture content reached 34.14%. It was then powdered and passed through a 850 µm mesh sieve. In order to remove the fats, waxes and resins, the screened powder was macerated in petroleum ether for 12 h so that these substances will not disturb the extraction process. The resulted powder was dried under a hood, and then extraction was conducted using 99.7% ethanol with the ratio of 1:10 (w/w) on an electrical stirrer at ambient temperature in darkness for 24 h. The extract was filtered using a Whatman filter paper 1, and the solvent was removed using a vacuum rotary evaporator at 40°C; hence, the extract got concentrated and kept at 4°C in a dark place until use [18].

Preparation of the dried residue of red grape: The residue obtained from the juicing of grape (*Vitis vinifera* L. var. Siahe Sardasht) was provided from Azarkam Co. Urmia, Iran. It was dried for 6-12 h in a countercurrent tunnel drier at 40-50°C until its moisture content reached 11.15%. Then it was powdered using an industrial mill and screened with a 250 µm mesh sieve. Next, it was irradiated in the atomic energy organization (Tehran, Iran) with the dose of 25 KGRY in order to eliminate all microbial contaminations [19] and kept at 4°C in a dark place until use.

Microbial analysis of the barberry extract and the dried residue of red grape

Preparation of the microbial strain and its growth: The lyophilized strains of *S. aureus* (ATCC 29213)

and *E.coli* (ATCC 25922) were purchased from the Iranian Research Organization for Science and Technology. These two bacteria were cultured in Mueller Hinton Agar (MHA), and incubated at 37°C for 24 h to generate a distinct colony. Next, a suspension of the bacteria equivalent to 0.5 McFarland (containing 9.95 ml of sulfuric acid 1% and 0.5 ml of barium chloride 1.175% aqueous solution) including 1.5 * 10^8 bacterial cell/ml was prepared [20].

Determination of the barberry extract MIC: The MIC of the barberry extract was measured through the agar dilution method. 1-5 mg/ml dilutions of the barberry extract together with 5 ml of dimethyl sulfoxide (DMSO) 10% were prepared and sterilized by passing through a 0.45 µm filter. Then 1 ml of this solution was added to 20 ml of MHA and stirred using a tube shaker. After homogenization, the medium was poured into plates and solidified. Inoculation was performed through the spotting method. To do so, 1 µl of the 0.5 McFarland microbial suspension was transferred into the center of the plate using a micropipette. After 30 min, the plates containing *S. aureus* and *E. coli* were incubated at 37°C for 16-20 h. Checking the growth of the samples was done through comparison to the blank. During the MIC experiments, the microorganism without the presence of the anti-microbial component was used for the positive control, and the sterilized DMSO was employed for the negative control [21].

Determination of the MIC of the dried residue of red grape: Likewise, the MIC of the dried residue of red grape was measured through the agar dilution method. 1-10 mg/ml dilutions of the dried residue of red grape in addition to 5 ml of sterilized distilled water were prepared. Afterwards, 1 ml of this mixture was added to 15 ml of MHA, and vortex was carried out. After homogenization, the medium was poured into plates and allowed to solidify. Then inoculation was conducted through the spotting method. For this purpose, 1 µl of the 0.5 McFarland microbial suspension was transferred into the center of the plate using a micropipette, and after 30 min, the plates including *S. aureus* and *E. coli* were incubated at 37°C for 16-20 h. Checking whether the samples grew or not was performed by comparing with the blank. During the MIC experiments, the microorganism without the presence of the anti-microbial component was used for the positive control, and the sterilized distilled water was applied for the negative control [21].

Sausage manufacture: A total of 21 sausage samples were produced containing different amounts of nitrite, barberry extract and dried residue of red grape (Table 1). The samples were prepared in Solico meat products Co (Tehran, Iran). The sausage containing 55% meat was produced according to the Iranian commercial formulation. First, the farsh consisting of 55% meat (calf head and neck) and the other ingredients, including ice and water 15.87%, null flour 2%, wheat starch 3%, gluten 2.3%, soybean isolate 3%, spice, essential oil, ascorbic acid 1.25%, salt 0.9%, phosphate 0.3%, sugar 0.7%, frozen garlic 1%, and oil 15.3% was manufactured. These ingredients were mixed using a cutter (Seydelmann Aalen, Germany), and the produced farsh was mixed with nitrite, the barberry extract, the dried residue of red grape, and half of the remaining water and ice in separate batches. The resulted farsh was filled in suitable wraps by automatic filler, and placed in the cooker. The sausage samples were stored in the cooker at 75°C for 30 min so that the temperature of the sausage cold point reached 72°C. Thereafter, the samples' temperature was reduced with cold water. Finally, the samples were stored at 4°C for 30 days.

Table 1. Experiments generated and randomized positions in RSM

Run	Dried residue of red grape (%)	Barberry extract (mg/kg)	Sodium Nitrite (mg/kg)
1	2	0	60
2	2	600	60
3	0	600	60
4	0	0	120
5	0	300	90
6	0	300	60
7	0	600	30
8	2	300	30
9	1	0	90
10	1	0	60
11	1	600	90
12	2	0	30
13	2	300	90
14	1	300	60
15	1	0	30
16	1	300	60
17	1	600	30
18	0	300	30
19	2	300	60
20	0	0	60
21	0	0	0

Microbial tests of the sausage samples: 25 g of the sausage sample centroid was isolated and mixed with 225 ml of peptone water (0.1%) in a special sack (zip pack) stomacher (Lab blender, Seward Medical, London, UK). The mixture was homogenized with the speed of 400 rpm for 2.5 min. The homogenized samples were diluted in the tubes containing peptone water (0.1%) and the selective medium of each bacterium was used for its identification and counting [22]. All microbial tests were conducted after 30 days of storage of the samples at 4°C.

Total viable count: The prepared dilutions were cultured on the medium of plate count agar (PCA) through the method of pour-plate, and counted after 48 h of incubation at 35°C. The number of the bacteria were reported as $\log_{10}{}^{cfu}/_{gr}$ [22].

Tracking the presence and counting of *C. perfringens*: The prepared dilutions were cultured on the medium of sulfite polymyxin sulfadiazine (SPS) agar, vacuum-incubated at 37°C for 48-72 h, and then counted. The number of the bacteria were expressed as $\log_{10}{}^{cfu}/_{gr}$ [23].

Total psychrotrophic viable count: The prepared dilutions of the samples were cultured on the PCA medium through the method of surface culture. The plates were then placed in a refrigerated incubator at 7°C for 10 days. The number of the colonies were counted and recorded [22].

Optimization: The aim of optimization through RSM is to find the desirable point within the design space. Depending on the purpose of the process, this point could be a maximum, minimum or a region in which the response is constant within the entire range of the variables. Optimization was performed according to minimization of the microbial load, remaining nitrite and fat oxidation, as well as maximization of redness (a*), the ratio of redness to yellowness (a*/b*), hardness, overall acceptance, brightness and yellowness within the constant range. The optimum conditions of the processed samples were determined based on the desirability function. The optimal conditions were found to be 76.27 mg/kg of nitrite, 600 mg/kg of the barberry extract, and 0.2% of the dried residue of red grape.

Statistical analysis: A central composite design (CCD) was applied to investigate the effects of the three factors, namely the dried residue of red grape (%), the barberry extract (mg/kg) and nitrite (mg/kg), on the quantitative and qualitative attributes of the fine product (sausage). All experiments were triplicated, and the obtained data were analyzed through regression and the analysis of variance (ANOVA) by Design Expert 7. The error level of type I was considered as 0.05.

The analysis of the obtained data concerning the presence and counting of *C. perfringens* was carried out through the repeated measures analysis of variance (ANOVA) using the SPSS software (ver. 21). The data mean comparison was conducted using the Bonferroni`s multiple range test in the probability level of 0.05. All experiments were triplicated.

Inoculation and counting of *C. perfringens*: After the transfer of the lyophilized *C. perfringens* (ATCC 13124) to the medium of FTG, the suspension of the vegetative cells was acquired and spore formation was done in the medium of Duncan-Strong (DS). The suspension of the spores was transferred into micro-tubes and centrifuged to separate the medium. The suspension was rinsed twice, resuspended in peptone water (0.1%), and kept at 4°C. Before inoculation, 0.5 McFarland solution of the prepared suspension was thermally shocked at 80°C for 15 min, and the dilution of 10^2 was inoculated to the farsh of the control, blank and optimum samples and mixed by hand. Then the farsh was filled into the wraps and cooked at 75°C for 1 h, and cooled down with cold water. Counting of *C. perfringens* was implemented based on the method cited by Yetim et al. (2006) at 4°C during 30 days of storage.

Results

The MICs of the barberry extract and the dried residue of red grape on E. coli and S. aureus are summarized in Table 2. As observed, the anti-microbial effect of the barberry extract (3 and 4 for S. aureus and E. coli, respectively) is more than that of the dried residue of red grape (6 and 7 for S. aureus and E. coli, respectively). Both of them had larger anti-microbial effects on the Gram-positive S. aureus; in other words, the Gram-negative E. coli had a greater resistance to the anti-microbial effect of the barberry extract and the dried residue of red grape.

Table 2. The results of the MICs of barberry extract and dried residue of red grape on *E. coli* and *S. aureus*

Microorganism Samples	*Staphylococcus aureus*	*Escherichia coli*
Barberry extract(mg/kg)	3	4
Dried residue of red grape(%)	6	7

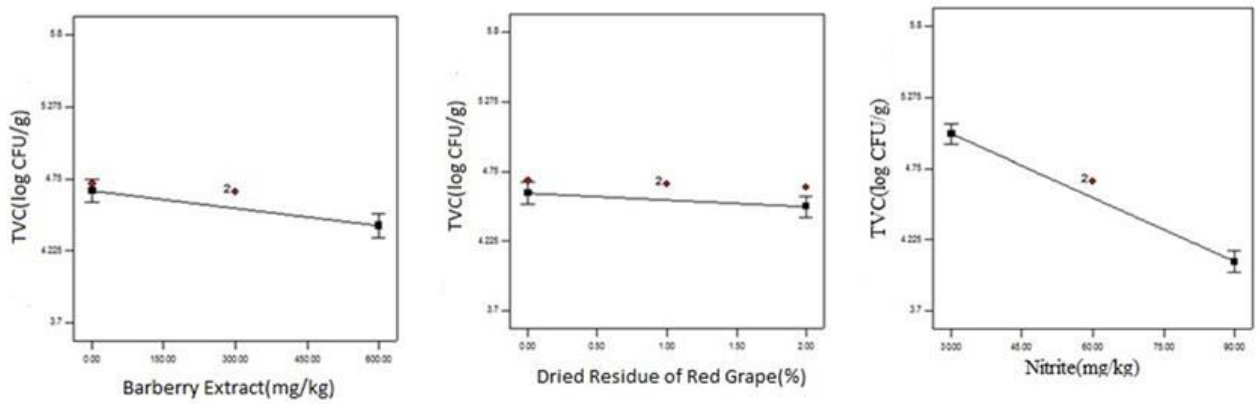
All standard deviations are equal to zero.

In the results of the response surface model, the calculated p-value for the measured microbial load after 30 days of storage at 4°C was less than 0.0001, indicating the significance of the model. The determination coefficient, R^2, (0.9073) and the adjusted determination coefficient, R^2_{adj}, (0.8909) confirmed that the model was very significant. In other words, 90.73% of the response variations are explained by the variations within the independent variables, and merely 9.27% of the response variations cannot be justified by the model. The adequate precision of the range of the predicted results within the design space is with the mean predicted error. This statistic measures the signal to noise ratio (SNR). The SNR greater than 4 is suitable; the SNR of this model was equal to 27.352, which is very nice for this statistic. The model terms with p-values higher than 0.1 are not significant. The significant terms of the model included the linear terms of the amounts of the barberry extract (B) and nitrite (C). The variance coefficient of 3.43% revealed the relatively low deviation of the experimental results from the predicted ones and the high confidence level of the data accuracy. The model for estimation of the total count of microorganisms with regard to the calculated regression coefficients is demonstrated in Equation 1 (A: dried residue of red grape, B: barberry extract, C: nitrite):

$$R = 4.54 - 0.051\ A - 0.13\ B - 0.047\ C \qquad \text{Eq. 1}$$

As depicted in Figures 1B and 1C, it is evident that as the barberry extract and nitrite increased, the microbial load decreased significantly. Although the dried residue of red grape caused the microbial load to decrease, its linear term had no significant effect on the response (Figure 1A). No *C. perfringens* was observed in all the tested samples during storage, and the psychrotrophic bacteria were not countable until the 30th day of storage.

The effects of the red barberry extract, the dried residue of red grape and nitrite on the optimum, control (120 mg/kg of nitrite) and blank (nitrite-free) samples during 30 days of storage at 4°C are presented in Table 3. The spores of Clostridium had no vegetation and growth in the optimum and control samples during storage. As observed in Table 3, vegetation and growth follow an incremental trend in the blank sample during the first 15 days of storage, and an obvious reduction is seen in the viable cell count at the end of storage (the 30th day).



A B C

Figure1. The effect of dried residue of red grape (A), barberry extract (B) and nitrite (C) on total viable counts (CFU/g) in the level of 0.05.

Table 3. The effect of barberry extract, dried residue of red grape and nitrite on the population of *C. perfringens* in sausage during 30 days of storage at 4°C

Samples	Day of storage		
	0	15	30
Control	ND	ND	ND
Blank	1.86±0.09[b]	2.79±0.15[a]	1.69±0.05[b]
Optimized	ND	ND	ND

The same letters in each row show the non-significant difference in the probability level of 0.05 ($p>0.05$) in Bonferroni's test (n=3 replications) in repeated measures analysis of variance (ANOVA). Control and blank samples with 120 and 0 mg/kg nitrite, respectively, and optimized sample with 76.27 mg/kg of nitrite, 600 mg/kg of the barberry extract and 0.2% of the dried residue of red grape.
ND: Not Detected

Discussion

The difference between the cell walls of Gram-positive and Gram-negative bacteria is the reason of the larger resistance of *E. coli* as compared to *S. aureus*. The two-layered and more complicated cell walls of Gram-negative bacteria are capable of reducing the diffusion and influence of antibiotic and anti-microbial substances rather than those of Gram-positive bacteria. Gram-positive bacteria have just a peptidoglycan layer in their cell walls. On the contrary, in addition to this internal peptidoglycan layer, Gram-negative bacteria have an external layer comprised of lipoprotein, phosphoprotein and protein, which causes the reduction of the effect of barberry extract and dried residue of red grape on Gram-negative bacteria [18].

Khaleghi (2011) studied the anti-microbial effect the ethanolic extract of blackberry on *S. aureus* and *E. coli*. He reported the MIC for *S. aureus* and *E. coli* as 0.78 and 1.625 mg/ml, respectively [24].

Mollaei et al. (2010) declared that alkaloids, especially berberine, bring about the anti-microbial effect of the barberry extract. They investigated the anti-microbial effects of the red and black berries on *S. aureus* and *E. coli* and found that the anti-microbial effect of the blackberry extract was more than that of the red berry extract, probably due to the difference in their composition, which in turn depends on the race, climate and the region of growth [18].

Kosalec et al. (2009) examined the anti-microbial effect of the root of red barberry (B.vulgaris) on *S. aureus*, and reported a considerable anti-microbial effect with the inhibition zone in the range of 9-16 ml meter [25].

Li et al. (2008) scrutinized the anti-microbial effects of six types of plants applied in traditional medicine on some Gram-positive and Gram-negative bacteria. They realized that the stem and leaf extracts of *Mahonia fortune* and the stem extract of *Mahonia bealei* had the strongest effect on *S. aureus* with the inhibitory diameter of more than 20 mm followed by the stem extract of *Berberis thunbergii* with the inhibitory diameter of 19.8 mm [26]. Owing to the existence of many different chemical groups, the anti-microbial effect of an extract cannot be distinguished by a single mechanism [27]. In addition, these phenolic compounds, existing abundantly in the extract, have anti-microbial effect through various mechanisms including the change in the diffusivity of the microbial cell, interfere with the membrane agents (such as electron transfer and loss of nutrients), interfere in the synthesis of protein and nucleic acid and enzymatic activity, interaction on the membrane proteins (which alters their structure and function), and the replacement of alkyl with the phenolic nucleus [28].

Up to now, no research has been done on the anti-microbial effect of the dried residue of red grape. Yet, Kao et al. (2010) evaluated the effects of grape seed extract on 21 bacterial species, and maintained that this extract was more effective on Gram-positive bacteria, especially the Cocci ones, rather than the Gram-negative ones, and the grape seed extract could likely be applied as an appropriate effective natural substitute to control the Staphylococcus food poisoning with suitable safety [29].

The results of the research performed by Ahn et al (2007) demonstrated that the commercial extract of grape seed (1%) reduced the number of *E. coli* and *Salmonella typhimurium* effectively [30].

The anti-microbial effect of the grape seed extract is principally attributed to the hydroxyl groups of the phenolic compounds such as Gallic acid (GA), para-coumaric acid, ferulic acid, caffeic acid, catechin, epicatechin, epigallocatechin gallate, courtin, kaempferol, murin, trans-resratol, cyaniding 3-glucosidase, and polymeric pro-anthocyanidins [31]. Phenolic compounds are able to diffuse into the bacterial cell walls, and react with the cytoplasmic proteins. This mechanism is obvious in the grape seed extract because of the non-ionization of phenolic acid [32].

Some specific microbial strains, which could contaminate meat and meat products through environment, are regarded as indicators of microbial spoilage during storage; a number of these strains were investigated in this study [22, 33]. Essence, extracts and herbal compounds contain complicated ingredients. When used in combination with each other, they could blurt additive, synergistic or antagonistic effects [34]. The anti-microbial

strength of the barberry extract could be ascribed to the bioactive compounds occurring in it, especially alkaloids and phenolic compounds [34]. Various mechanisms have been stated for the expression of the anti-microbial effect of nitrite including the reaction of nitrite and nitro acid with the SH groups of the bacterial cell walls [35], the effect on DNA and gene expression, and the destruction of the cell membrane and wall [36]. The anti-microbial effect of the herbal essences and extracts are more than that of plants and herbal fibers. Viuda-Martos et al. (2010b) attributed the anti-microbial effect of orange dietary fiber in sausage to the bioactive compounds existing in it, especially phenols and terpenes [37]. pH is one of the influential internal factors on the growth of microorganisms [38]. Due to its low pH (3.81), the dried residue of red grape had the pH of the samples decreased; preventing the growth of microorganisms to some extent. The anti-microbial effect of the dried residue of red grape was not observed, probably because of its low amount. *C. perfringens* was not observed, may be due to the small microbial population of the raw meat, cooking conditions, and suitable storage.

Psychrotrophic bacteria are referred to as a group of various bacterial strains; although their optimum temperature of growth and metabolic activity ranges from 20 to 30°C, they are capable of growing at 7°C or less [39]. These bacteria are the most important bacterial category in developing the spoilage of meat products [40]. Nevertheless, psychrotrophic bacteria were not countable after 30 days of storage which is due to the predominance of Gram-negative bacteria in this group and the larger effect of the cooking temperature (pasteurization) on Gram-negative bacteria, as well as the reduction of the thermal strength of microorganisms by some components present in the formulation such as phosphate and glutamate. The results of the present study conformed to those presented by Viuda-Martos et al. (2010b) about the effects of the orange fiber, the oregano essence and the rosemary essence on Mortadella at 4°C. In that study, thermal processing, aseptic slicing and occurrence of salt in the product were the possible reasons the psychrotrophic bacteria did not grow during storage (24 days).

The results of the incremental or synergistic effect of the barberry extract, the dried residue of red grape and nitrite on *C. perfringens* in the samples were consistent with the results of Zarringhalami et al. (2009) who examined the effects of the annatto extract and nitrite, and Marefian et al. (2009) who investigated the effect of cinnamon extract on *C. perfringens* in sausage. The incremental trend of the growth and vegetation of *C. perfringens* during the first 15 days of storage could be justified by the rich nutritional content, which is desirable for the growth of the target microorganism, as well as the instability of the sample temperature,[36]. *C. perfringens* grows in the temperature range of 15-50°C. Moreover, spore vegetation, aided by the high storage temperature, could lead to the population growth. According to Scott et al. (2001) *C. perfringens* is a psychrolytic spoilage-generating bacterium that ferments carbohydrates. The final products of fermentation containing butyric and acetic acids reduce the pH of the medium. Sausage has a considerable content of fermentable carbohydrates (sugar and starch), which are metabolized by many inoculated cells of *C. perfringens* in the food model leading to acid production and hence pH reduction. Furthermore, the likelihood of the growth of thermo-tolerant microbes- namely the remaining cells after the cooking process, which are naturally present in meat and meat products, produce anti-bacterial agents such as bacteriocins, anti-microbial peptides, ethanol, peroxide and organic acids, resulting in a decrease in the number of *C. perfringens* [41]. The stability of the storage temperature (4°C) could also be another reason for the reduction in the growth of *C. perfringens* during the second 15 days of storage. The inhibitory effect of nitrite on *C. perfringens* could be due to the reaction of nitrite and nitro acid with the SH groups in the bacterial cells preventing the activity of enzymes including glyceraldehyde 3-phosphate dehydrogenase. In Clostridium, nitrite reacts with the iron/sulfur groups of certain proteins like ferredoxin, to form iron/nitrous oxide complexes; this restrains the phosphoroclastic system, involving the transformation of pyruvate to acetyl phosphate, electron transfer and ATP formation [36].

Conclusion: According to the MIC values of the barberry extract and the residue of red grape, an antibacterial activity was observed against the tested bacteria. We can conclude that the optimized sample of inoculated C. *perfringens* was acceptable after 30 days of storage. This result also suggests using the combination of the barberry extract, dried residue of red grape, and reduced level of nitrite for controlling the microorganisms' growth, and producing a healthier product in order to potentially reduce the risk of cancer due to reduced nitrosamine formation in sausage products.

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References

1. Indumathi J, Moorthy P, Reddy K. S & Babu A. J. Development of low-Fat Chevon Patties Using Sodium Alginate as Fat Replacer. TNJVAS 2011;7:167-173.
2. Ruiz-Capillas C, Herrero A, Tahmouzi S, Razavi S, Triki M, Rodriguez-Salas L, Samcová K & Jiménez-Colmenero F. Properties of reformulated hot dog sausage without added nitrites during chilled storage. Food Sci Technol Int 2014; 0:1-10.
3. Juneja V. K, Marks H & Thippareddi H. Predictive model for growth of Clostridium perfringens during cooling of cooked ground chicken. IFSET 2009; 10: 260-266.
4. Labbe R. G & Juneja V. K. Clostridium perfringens. Foodbone Diseases. Academic Press, Amsterdam, Boston, London, New York 2002: 119-126.
5. Labbe R & Huang T. Generation times and modeling of enterotoxin-positive and enterotoxin-negative strains of Clostridium perfringens in laboratory media and ground beef. J Food Protec 1995; 58:1303-1306.
6. Petit L, Gibert M & Popoff M. R. Clostridium perfringens: Toxinotype and genotype. Trends microbiol 1999; 7: 104-110.
7. Cassens R. G. Composition and safety of cured meats in the USA. Food Chem 1997; 59: 561-566.
8. Honikel K.-O. The use and control of nitrate and nitrite for the processing of meat products. Meat Sci 2008; 78: 68-76.
9. Jafari M & Emam-Djomeh Z. Reducing nitrite content in hot dogs by hurdle technology. Food Control 2007; 18: 1488-1493.
10. Zarringhalami S, Sahari M & Hamidi-Esfehani Z . Partial replacement of nitrite by annatto as a colour additive in sausage. Meat Sci 2009; 81: 281-284.
11. Viuda-Martos M, Fernández-López J, Sayas-Barbera E, Sendra E, Navarro C & Pérez-Álvarez J. Citrus Co-Products as Technological Strategy to Reduce Residual Nitrite Content in Meat Products. J Food Sci 2009; 74: R93-R100.
12. Lee H. Y, He X & Ahn J. Enhancement of antimicrobial and antimutagenic activities of Korean barberry (Berberis koreana Palib.) by the combined process of high-pressure extraction with probiotic fermentation. J Scie Food Agri 2010; 90: 2399-2404.
13. Yi Z.-B, Yu Y, Liang Y.-Z & Zeng B. Evaluation of the antimicrobial mode of berberine by LC/ESI-MS combined with principal component analysis. J Pharm Biomed Anal 2007; 44: 301-304.
14. Freile M, Giannini F, Pucci G, Sturniolo A, Rodero L, Pucci O, Balzareti V. & Enriz R. (2003). Antimicrobial activity of aqueous extracts and of berberine isolated from Berberis heterophylla. Fitoterapia 2003; 74: 702-705.
15. Pourali A, Afrouziyeh M & Moghaddaszadeh-ahrabi S. Extraction of phenolic compounds and quantification of the total phenol of grape pomace. Eur J Exp Biol 2014; 4: 174-176.
16. Jayaprakasha G, Selvi T & Sakariah K. Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts. Food Res Int 2003; 36:117-122.
17. Su Cha D, Choi J. H, Chinnan M. S & Park H. J. Antimicrobial films based on Na-alginate and κ-carrageenan. LWT-Food Sci Technol 2002; 35: 715-719.
18. Mollaei A, Rezaei K, Khodayian F & Razavi S. Segregate functional compound from barberry's fruit extracts. Iranian National Functional Food Conference 2010; 341-350.
19. Perez MB B.S, Croci CB. Retention of antimicrobial activityin gamma irradiated argentinian sage and oregano. Food Chem 2011; 6: 121-126.
20. McFarland J. The nephelometer: An instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. JAMA 1997; 49: 1176-1178.
21. Collins C.H, Grange J.M, Lyne P.M, Falkinham J.O. Collins & Lyne's Microbiological methods. CRC Press; 8th edition (March 26, 2004), chap 12:177-196.
22. Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J & Pérez-Álvarez J. Effect of added citrus fibre and spice essential oils on quality characteristics and shelf-life of mortadella. Meat Sci 2010; 85: 568-576.
23. Yetim H, Kayacier A, Kesmen Z & Sagdic O. The effects of nitrite on the survival of Clostridium sporogenes and the autoxidation properties of the Kavurma. Meat Sci 2006; 72: 206-210.
24. Khaleghi A. Replacing of nitrite with berberis crataegina extract in cocked beef sausage, MSc thesis 2011, Sari University.
25. Kosalec I, Gregurek B, Kremer D, Zovko M, Sanković K & Karlović K. Croatian barberry (Berberis croatica Horvat): a new source of berberine analysis and antimicrobial activity. World J Microbiol Biotechno 2009; 25: 145-150.
26. Li A, Zhu Y, He X, Tian X, Xu L, Ni W& Jiang P. Evaluation of antimicrobial activity of certain Chinese plants used in folkloric medicine. World J Microbio Biotechno 2008; 24: 569-572.
27. Moarefian M, Barzegar M & Sattari M. Cinnamomum zeylanicum essential oil as a natural antioxidant and antibactrial in cooked sausage. J Food Biochem 2013; 37: 62-69.
28. Zhang W, Xiao S, Samaraweera H, Lee E. J & Ahn D. U. Improving functional value of meat products. Meat Sci 2010; 86: 15-31.
29. Kao T.-T, Tu H.-C, Chang W.-N, Chen B.-H, Shi Y.-Y, Chang T.-C & Fu T.-F. Grape seed extract inhibits the growth and pathogenicity of Staphylococcus aureus by

interfering with dihydrofolate reductase activity and folate-mediated one-carbon metabolism. Int J Food Microbial 2010; 141: 17-27.

30. Ahn J, Grün I.U. & Mustapha A. Effects of plant extracts on microbial growth, color change, and lipid oxidation in cooked beef. Food Microbiolo 2007; 24: 7-14.
31. Adams B, Sivarooban T, Hettiarachchy N & Johnson M. Inhibitory activity against Listeria monocytogenes by soy protein edible film containing grape seed extract, nisin, and malic acid. Discovery 2005; 6: 3-9.
32. Corrales M, Han J H & Tausche B. Antimicrobial properties of grape seed extracts and their effectiveness after incorporation into pea starch films. Int J Food Sci Tech 2009;44: 425-433.
33. Tajik H, Farhangfar A, Moradi M & Razavi Rohani S. M. Effectiveness of clove essential oil and grape seed extract combination on microbial and lipid oxidation characteristics of raw buffalo patty during storage at abuse refrigeration temperature. J Food Process Preserv 2014; 38: 31-38.
34. Fu Y, Zu Y, Chen L. Shi X, Wang Z, Sun S & Efferth T. Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytother Res 2007; 21: 989-994.
35. Riha W. E & Solberg M. *Clostridium perfringens* inhibition by sodium nitrite as a function of pH, inoculum size and heat. J food sci 1975; 40: 439-442.
36. De Oliveira T.L. C, de Araújo Soares R, Ramos E. M, das Graças Cardoso M, Alves E & Piccoli R. H. Antimicrobial activity of Satureja montana L. essential oil against *Clostridium perfringens* type A inoculated in mortadella-type sausages formulated with different levels of sodium nitrite. Int J Food Microbiol 2011; 144: 546-555.
37. Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J & Pérez-Álvarez J. Effect of orange dietary fibre, oregano essential oil and packaging conditions on shelf-life of bologna sausages. Food Control 2010; 21: 436-443.
38. Jay J. M, Loessner M. J & Golden D. A. Modern food microbiology 2005. Springer.
39. Samaržija D, Zamberlin Š & Pogačić T. Psychrotrophic bacteria and milk and dairy products quality. Mljekarstvo 2012; 62:77-92.
40. Davies A. R & Board R. G. Microbiology of Meat and Poultry.1998. Springer.
41. Scott V.N, Anderson J.E, Wang G. Mesophilic anaerobic sporeformers, In: Downes, F.P., Ito, K. (Eds.), Compendium of Methods for the Microbiological Examination of Foods, 4th ed. American Public Health Association, Washington 2001; 325–330 (chap. 34).