ISSN 2250-0480 Special Issue SP 2016 – Issue 2 P.No. 39 to 48

International Journal of Life science & Pharma Research

RECOVERY OF *BACILLUS COAGUALNS* AS A PROBIOTIC SPOREFORMER IN THE RAW BATTER OF COCKTAIL SAUSAGE AS INFLUENCED BY CHOPPING, FORMULATION AND SURFACTANT

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ABSTRACT

The aim of the present study was to investigate the influence of chopping, formulation and surfactant on the viability and recovery of *Bacillus coagulans* spore in the raw batter of cocktail sausage. The formulation caused a 2.5-2.7 log CFU/g reduction in the enumeration of *B. coagulans* spore in all the raw batters of cocktail sausage (40, 55, and 70%) studied; however, no statistically difference was observed for the viable counts of *B. coagulans* spore among their batters (p>0.05), inferring that the effects of determinant factors like salt, nitrite, phosphate and ascorbate on the spore germination and outgrowth were virtually the same due to their similar amounts in all the formulations. As it was expected, the chopping step in the sausage manufacture could significantly increase the number of B. *coagulans* spores to 1.2, 1.9 and 2.1 % in the raw batter of 40, 55, and 70% cocktail sausage, respectively (p<0.05). Results also showed that the addition of surfactant (Tweens 20 and 80) at 0.05 % (v/v) to the spore solution of 40% cocktail sausage significantly reduced the formation of the spore clumps; thus caused homogenous distribution and also increase (on average) the enumeration of *B. coagulans* spore in all three random sampling places of the cutter compared to the control (p<0.05).

Keywords: *B. coagulans*; Chopping; Cocktail sausage; Probiotic sporeformers; Surfactant

INTRODUCTION

Probiotics are defined as live microorganisms that in adequate amounts produce health benefits on the host [1]. Several health benefits are attributed to the ingestion of foods containing probiotic cultures [2]. Some of them proven scientifically and others still requiring further studies in humans [3]. Probiotic food is defined as a food product that contains viable probiotic microorganisms at adequate levels incorporated in a suitable matrix [3, 4]. This means that they must maintain their viability and metabolic activity for the duration of the shelf-life of the product, from their production up to their ingestion by the consumer, and also that they must be able to survive in the gastrointestinal tract [5]. The viability of probiotics has been both a marketing and technological concern for many industrial producers. However, there is no worldwide consensus regarding on the minimum amount of probiotic microorganisms to be ingested to ensure their functionality and it will vary as a function of the strain and the health effect is desired [6]. Nevertheless, assuming a daily consumption to 100 g or 100 ml, populations of 10^6–10^7 CFU g^{-1} or mL^{-1} in the final product are established as minimum daily therapeutic quantities of probiotic cultures in processed foods [7, 8], reaching 10^8–10^9 CFU, hence benefiting human health [9]. However, factories are interested in formulating products with probiotics which are recognizing that probiotic microorganisms must not only be supported by solid clinical data. But it is alo important that the probiotic must yield health benefits and they should they should survive throughout the manufacturing process. In addition, the shelf life of the product and ultimately survive the passage through the digestive system is very important [10, 11]. Unlike the traditional probiotic bacteria such as special species of the genera *Lactobacillus* and *Bifidobacterium*, spore probiotics (as novel probiotics) are able to resist the harsh manufacturing process, storage and shipping conditions, acidity of the stomach and bile acids [12]. Out of more than 100 *Bacillus* spp. known, only a few (including *B. coagulans*) have been most extensively examined and are being used as probiotics for human consumption [13, 14]. On the other hand, although the use of probiotics has found its way into the dairy sector and they are now mainly incorporated into fermented dairy products, through use of lactic acid bacteria (LAB). Thus, potential opportunities are there of meats and meat products being used as a functional food which yet remain to be explored. In fact, the incorporation of probiotic bacteria to meat products could bridge the existing gap in the field of probiotic products of animal origin also. Moreover, it seems that it would be a simple way to reduce negative nutritive image of these products due to their fat content and the use of preservatives and additives in their formulation [15, 16]. Bacterial spores like spore probiotics commonly require activation including heat treatment at a time and sublethal temperature appropriate to the organism concerned to finish off dormancy and enable rapid germination to occur [17]. The amount of heat shock necessary to activate spores optimally varies greatly with the different organisms, varying from species to species and even very much strain dependent, the medium and its composition (i.e. ingredients and final pH) in which spores are suspended and, if germinants are present, the particular germinants and their concentrations [18]. Importantly, how microorganisms are physically distributed in a food, determines the value of the data on prevalence and concentration obtained through sampling and testing [19]. It has been observed that bacterial spores (including spore probiotics) tend to form clumps (or auto-aggregation) in suspensions which lead to an uneven distribution of spore probiotics throughout food matrices. It was shown that the formation of spore clumps was dependent on the increase of the hydrophobicity of spore surface [20, 21]. It has been reported that, the increase in the hydrophobicity of bacterial spore is due to denaturation of the surface proteins [20] and it was also hypothesized that the addition of surfactant to a spore suspension may decrease the hydrophobicity of spores which, decrease the possible contribution of spore clumping, and thereby increase the 'recoverable' or 'retrievable' spores, compared to the initial count [20, 22]. In addition, mechanical

mixing (like the chopping and/or comminution step in sausage manufacture) results in dispersion and rearrangement of spores in a food product, though further development of the spore distribution depends somewhat on the consistency of the inoculated product. On the other hand, spore germination is mainly triggered by nutrient germinants like single amino acids and carbohydrates (sugars) that are specific to different types of spores. In addition to nutrients, spore germination can be triggered by nonnutrient agents, including lysozyme, cationic surfactants, ethanol, EDTA, NaCl (high concentrations), NO_2, and sorbate, and some physical processes (high hydrostatic pressure) [23, 24]. It appears that maintaining the maximum viability and the highest recovery of *Bacillus* spores is of utmost importance for the successful application of probiotic sporeformers in foods like meat products. In other words, inhibition of *B. coagulans* spore germination and outgrowth in all steps of cooked sausage manufacture including production, storage, and shipment till consumption time is a determinant factor in this regard. In the present study, the influence of chopping, various sausage formulations and their ingredients affecting spore germination, and addition of surfactant on the recovery of *B. coagulans* in the raw batter of cocktail sausage were investigated.

MATERIALS AND METHODS

Materials and chemical reagents

All the chemical and reagents used for the present study were of analytical grade and purchased from Merck (Darmstadt, Germany).

Bacterial spore and preparation of spore suspension

The bacterial spore used was *B. coagulans*, obtained from World from Natures Only, INC., USA. In order to assure the enumeration and purity of the spore, it was cultured in Müller-Hinton agar (Merck, Germany). Preparation and purification of the studied spore were done according to Alebouyeh et al. [25].

Heat shock and culture media

In this study, a heat shock of 68°C for 20 min and trypticase soy agar (TSA) medium (Difco, USA) yielding the highest recovery of *B. coagulans* spore were used based on our recent previous work [26]. In practice, a suspension of *B. coagulans* spore was placed in a 68± 1°C water bath (Memmert, Germany) for 20 min. After heat treatment, the suspension of the spore was immediately cooled down in a crushed ice water bath and then serially diluted and spread-plated using TSA. The inoculated plates were incubated for 2 days at 37^{o}C and then the colonies were manually counted from duplicate plating. In order to prevent an underestimation of the number of the examined spore, the time between preparation of the primary dilutions and the heat shock step was considered less than 10 min according to the study conducted by te Giffel et al. [27].

Cocktail sausage manufacture

The cocktail sausages, containing 40, 55, and 70 % meat contents, were made in Gooshtiran meat products factory. Frozen commercial beef cut (15% fat) and vegetable oil were purchased from a local supplier. Frozen meat packages were thawed for approximately 18 h at 4°C. The ingredients of the sausage mass and their relative proportions are given in Table 1. Build up method among different chopping techniques was selected for preparation of the sausage batter [28].

Table 1
The ingredients of the sausages prepared for this study

Ingredient	Amount (%)		
	40% cocktail sausage	55% cocktail sausage	70% cocktail sausage
Minced meat (beef [1])	40	55	70
Water (crushed ice)	22	20	13
Vegetable oil	19	10	4.5
Filler assortment [2]	12	8	5.5
Fresh garlic	1.8	1.8	1.8
Pasteurized whole egg	2.0	1.7	1.8
Salt (NaCl)	1.5	1.7	1.7
Polyphosphate	0.35	0.40	0.40
$NaNO_2$ (10% water solution)	0.012	0.012	0.012
Sodium ascorbate	0.04	0. 04	0.04
Sugar	0.20	0.25	0.25
Spice assortment	1	1	1

[1] Beef used for sausage manufacture was topside and shin cut (15% fat).

[2] including wheat flour, wheat starch, gluten, and texturized soy protein.

In practice, initially the minced meat was ground at low speed and blended with sodium nitrite, salt (NaCl), and polyphosphate respectively for about 2 min in a 10 kg silent bowel mini cutter (Allen, K21 Ras 83132, Germany). Half of the water with crushed ice was then added and mixed in the cutter at the high speed setting (2800 rpm). At that point inoculation of the spore solution shaken vigorously to the raw batter of the sausage was done. In order to prepare the spore solution, *B. coagulans* spores at an initial concentration of 15.18 log CFU/mL, were transferred to a screw-capped glass bottle containing 200 mL sterile deionized water. The final inoculum size added to the cutter bowel was 12.88 log CFU/10 kg. The oil was then added and chopped until the temperature of the mixture reached 8°C. The rest of the water and other ingredients were added and chopped for an additional 3.20 min, up to l2°C until they were evenly distributed and the emulsion was complete. A temperature probe (Kane-May, KM330, Harlow, Germany) was used to monitor the temperature of the emulsion, which was maintained below 12°C as the end point for chopping all the treatments during the batter preparation. Total emulsification time was about 8-10 min. The processing room temperature was about 17 ± 1°C and the final weight of each treatment was approximately 10 kg. Processing was repeated two times for each batch.

Addition of surfactant

Tween 20 and Tween 80 from Wako Pure Chemical, Osaka, Japan were separately added at 0.05 % (v/v) only to the spore solution of 40% meat cocktail sausage. In order to examine influence of the surfactants on the distribution of the examined spore in the raw sausage batter, samples were randomly taken from three different places far apart from each other in the bowel mini cutter.

Effect of formulation and chopping

To evaluate the effect of cocktail sausage ingredients, individually and in combination with chopping, on the viability and recovery of *B. coagulans* spore, two separate batches of raw sausage batter were prepared for each of the formulations (containing 40, 55, and 70 % meat contents) studied. One batch had only the cutter bowel rotation without the action of chopping knives, whereas another batch having the synchronous running of chopping knives and bowel rotation (both together). The samples from two separate batches were randomly taken 3 min after the addition of *B. coagulans* spore solution to the cutter bowel as per ISO 3100-1 [29]. Enumeration of *B. coagulans* spore was determined by viable plate count (CFU/g) on trypticase soy agar after heat shock at 68°C for 20 min. The plates were aerobically incubated for 48 hrs. at 37^{o}C and then colonies were manually counted. The numbers of the spore were calculated from duplicate plating.

Statistical analysis

All data are presented throughout as mean value with their standard deviation (mean ± SD) for each treatment. Colony-forming units (CFUs) in all experiments were converted to $\log_{10}$values. All Experiments were replicated. Statistical analysis of the data was carried out using Student's t-test and one-way analysis of variance (ANOVA) of SPSS (Version 20, SPSS Inc, Chicago, IL, USA). The differences among the mean values were detected with Duncan's Multiple Range test at a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

It should be mentioned that the properly dispersion of *B. coagulans* spores in sausage batter is vital for sampling precision and the minimum therapeutic amount of spore probiotics necessary per gram of sausages too. As shown in Table 2, the results of the present study revealed that addition of surfactants (Tweens 20 and 80) at 0.05 % (v/v) to the spore solution of 40% meat cocktail sausage caused homogenous distribution of the examined spore in all three random sampling places of the cutter compared to the control sample ($p<0.05$). In addition, both Tween 20 and Tween 80 could increase (on average) the enumeration of *B. coagulans* spore in all three random sampling places of the cutter. A possible explanation for the homogenous distribution and increase in the number of counted spores of *B. coagulans* due to the addition of the surfactants is that they help in dispersion of the spores through 'de-clumping', i.e., by breaking their aggregates [22].

Table 2

Influence of surfactant on viable spore count of B. coagulans dispersed in the raw batter of 40% cocktail sausage [1].

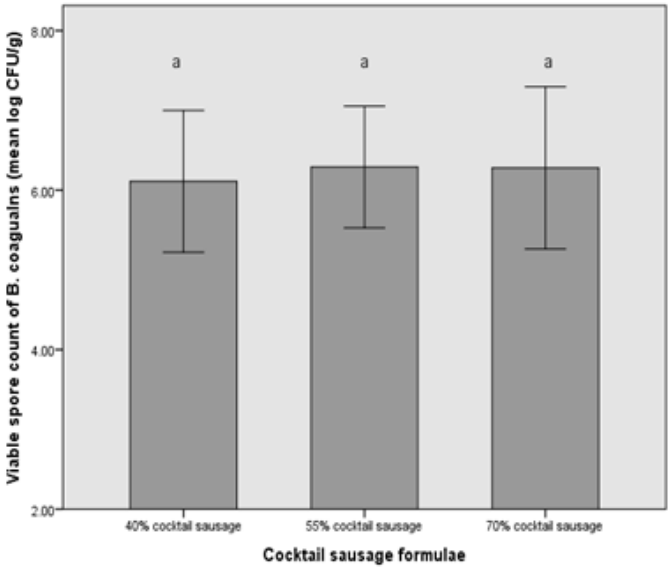
Treatments	Viable count (log CFU/g) of *B. coagulans* spore in different random sampling places (RSPs) of a cutter		
	RSP-1	RSP-2	RSP-3
Tween 20	9.60 ± 0.05^{A}	9.65 ± 0.06^{A}	9.74 ± 0.04^{A}
Tween 80	9.95 ± 0.03^{A}	9.93 ± 0.02^{A}	9.91 ± 0.01^{A}
Control	9.95 ± 0.03^{A}	9.71 ± 0.08^{AB}	9.39 ± 0.2^{B}

[1] Each value in the table is the mean ± SD of duplicate samples, obtained in two independent experiments. The initial inoculum of *B. coagulans* spore was 12.88 log CFU/10 kg. Enumeration of the spore was conducted after heat shocking at 68°C for 20 min, culturing in trypticase soy agar, and aerobically incubating at 37°C for 48 h, respectively. Tweens 20 and 80 were added at 0.05% (v/v). [A, B] Different capital letters in the same row denote significant difference ($P \leq 0.05$) among random sampling places for the same treatment. Thus, the

spores clinging together would be found in a single colony after germination and hence would be counted as one, after dispersion they will produce several colonies of their own and hence be counted as many [22]. Although Tween 80, compared to Tween 20, caused further increasing in the number (CFU/g) of *B. coagulans* spore in all three random sampling places of the cutter, it was not statistically significant (p>0.05). The key ingredients of cocktail sausage implicated in the spore germination and outgrowth of *B. coagulans* include sodium nitrite ($NaNO_2$), salt (NaCl), sodium ascorbate or erythrobate, solubilized myofibrillar proteins and acidulants like lactate and diacetate if used in the sausage formula [30]. Thus, it can be speculated that a 2.5-2.7 log reduction in the number of CFU of *B. coagulans* spore in all the raw batters of the examined cocktail sausage (40, 55, and 70%) was related to the impact of these ingredients (Fig. 1).

Figure 1

Real viable spore count of B. coagulans inoculated in the raw batter of cocktail sausage samples (by subtracting the total number (log CFU/g) of the inoculated spores from that of naturally existent Bacillus spores in the raw sausage matrix). Values with the same letter are not significantly different at p=0.05. Error bars indicate the standard errors of experiment.



Bell and De Lacy also showed that *Bacillus* spore germination was accelerated and enhanced by the presence of at least 2% NaCl [31]. However, increasing NaCl concentrations caused increasing, albeit reversible, inhibition of spore germination. High salinity delayed and increased the heterogeneity of germination initiation, slowed the germination kinetics of individual *Bacillus* spores and the whole spore population, and decreased the overall germination efficiency [32]. Again, sodium nitrite enhanced *Bacillus* spore destruction and increasing its concentrations, resulted in greater inhibition of spore formers germination and outgrowth. In addition, increasing the viable count of *B. coagulans* spore in the raw batters of 55 and 70% cocktail sausage (ca. 0.82 and 0.97 log CFU/g, respectively), compared to that in the raw batter of 40% cocktail sausage, was related to the contamination of cocktail sausage ingredients with *Bacillus* spores (Table 3). It is worth noting that the higher loads of *Bacillus* spore in the raw batters of 55% and 70% control sausages were related to the main sausage ingredient, i.e. meat content. In fact, the higher meat content in the sausage formula, the more contamination with *Bacillus* spores. However our results revealed that the viable counts of *Bacillus* spores between 55% and 70% control sausage were not significantly different (p>0.05) (Table

3). As shown in Figure.1, the results of this study confirmed that even though the amounts or concentrations of ingredients (especially meant content) were different in the formula of various cocktail sausages (40, 55, and 70%) prepared in this study, the viable counts of *B. coagulans* spore in their raw batters were not significantly different (p>0.05). The reason for this is that salt, nitrite, phosphate and ascorbate, as determinant factors in spore germination and outgrowth, had roughly the same amounts in all the studied formulations of cocktail sausage, so they had a similar effect on the viability and recovery of inoculated *B. coagulans* spores (p<0.05). The main determinant of structure in emulsion type sausages like cocktail sausages is the extent of comminution or chopping, that is, the size of the meat particles in the batter [33].

Table 3
Viable spore count (log CFU/g) of B. coagulans in inoculated and control raw batters of cocktail sausage [1].

Treatments [b]	Cocktail sausage formulae		
	40%	55%	70%
Inoculated with *B. coagulans* spores	9.70 ± 0.07^{a}	10.52 ± 0.13^{b}	10.67 ± 0.03^{b}
Control	3.55 ± 0.03^{a}	4.23 ± 0.18^{b}	4.39 ± 0.07^{b}

[1] values were determined after heat shock (68°C/ 20 min) from serially decimally diluted samples to approximately 10^3 CFU/mL in sterile 0.1% peptone water and then trypticase soy agar to give a statistically valid range of colonies; Mean ± SD values in the same row followed by the same letter are not significantly different (P > 0.05;). The initial concentration of the examined spore was 12.88 log CFU/10 kg. In addition, mixing or chopping is likely to lead to a more random spatial distribution of the microbial populations within the batch of sausage and a changing of the number of microorganisms per portion, for instance, per unit of weight [19]. Considering the tendency of spores like probiotic sporeformers to form clumps, it is clear that the role of chopping in dispersion of spores in the semisolid batter of cocktail sausage and movement or dislodging them from the surface and transferred to another area of the surface or to another part of the sausage batter are more vital than its action on vegetative cells.

Figure 2

Effect of formulation and chopping on the recovery (log CFU/g) of *B. coagulans* spore in the raw batter of cocktail sausage with different meat contents. Values were determined after heat shock (68°C/ 20 min) from serially decimally diluted samples to approximately 10^3 CFU/mL in sterile 0.1% peptone water and then on trypticase soy agar to give a statistically valid range of colonies; Mean ± SD values with different letters (a & b) are significantly different; $p<0.05$. The initial inoculum of *B. coagulans* spore was 12.88 log CFU/10 kg.

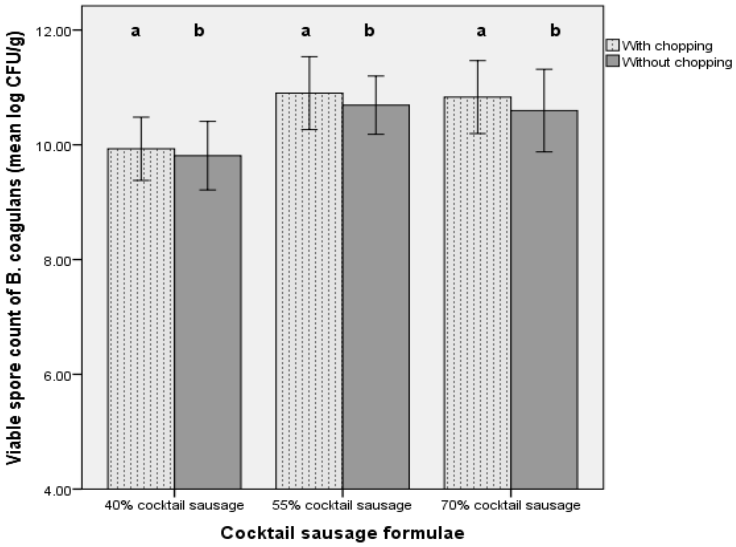


Figure. 2 depicts the effect of chopping step on dispersion and thereby the recovery of *B. coagulans* spores, through the course of producing a batch of cocktail sausage. As can be seen, chopping could significantly increase the enumeration of B. *coagulans* spores to 1.2, 1.9 and 2.1 % in the raw batter of 40, 55, and 70% cocktail sausage, respectively ($p<0.05$). Our results also showed that by raising the amount of meat in the sausage formulation, the chopping could enhance the recovery and concentration of B. *coagulans* spore ($p<0.05$).

CONCLUSION

The use of probiotic sporeformers like B. *coagulans* is in growing trend in the food market. Collectively, the present study clearly demonstrated that sausage ingredients especially those triggering and determining in spore germination and outgrowth, the chopping step in sausage manufacture, and surfactant affect on the recovery of the spore populations in the raw batter of cocktail sausage. As a consequence, in order to have a reliable sampling and the minimum therapeutic amount of probiotic sporeformers per gram of sausages and on the other hand, to obtain the maximum possible recovery of the spores, these main parameters have to be considered on a case-to-case basis.

ACKNOWLEDGEMENT

The paper has been taken from PhD dissertation of Food Science and Technology in Shahid Beheshti University of Medical Sciences. The authors would like to acknowledge Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences and Gooshtiran Meat Products Factory, Tehran, Iran for their important contributions to this research.

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