Asian Food Science Journal

2(3): 1-11, 2018; Article no.AFSJ.41374

Assessment of the Inhibitory Effect of Nisin (E234) on *Salmonella typhimurium* and *Bacillus subtilis* in Chicken Sausage

T. S. P. Jayaweera[1], J. M. C. S. Jayasinghe[1], D. N. N. Madushanka[1], D. G. Yasawathie[1] and H. A. D. Ruwandeepika[1*]

[1]Department of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, Belihuloya, P.O.Box 70140, Sri Lanka.

Authors' contributions

This work was carried out in collaboration between all authors. Authors TSPJ and HADR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JMCSJ, DNNM, HADR and DGY managed the analyses of the study. Authors JMCSJ and HADR managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AFSJ/2018/41374
Editor(s):
(1) Surapong Pinitglang, Assistant Professor, Department of Food Business Management, School of Science and Technology, University of the Thai Chamber of Commerce, Bangkok, Thailand.
(2) Marijana Sokolovic, Feed Analysis Laboratory, CVI - Poultry Centre, Zagreb, Croatia.
Reviewers:
(1) Raul, Ferrer-Gallegp, Spain.
(2) Maria de Lourdes Reyes Escogido, Universidad De Guanajuato, Mexico.
Complete Peer review History: http://prh.sdiarticle3.com/review-history/24861

Original Research Article

Received 7th March 2018
Accepted 16th May 2018
Published 29th May 2018

ABSTRACT

Aims: Nitrite is used as the main curing agent in the process of sausage production, but Nitrite has limited usage in food processing due to its, carcinogenic effect. Thus, much attention has been given to find alternative compounds to replace the Nitrite over the past decades. Nisin, a natural polypeptide compound extracted from *Lactococcus lactis* with no toxin production has been identified as a potential compound in this regard. This study aimed at assessing the inhibitory effect of nisin (E234) against *Salmonella typhimurium* and *Bacillus subtilis* and to investigate the potential of nisin to replace the antimicrobial property of Nitrite in broiler chicken sausage production.
Study Design: This is a laboratory-controlled experimental design.
Place and Duration of Study: The study was conducted at the Laboratory of Livestock Production, Faculty of Agricultural Sciences Sabaragamuwa University of Sri Lanka during the period of March

**Corresponding author: E-mail: ruwandeepika@yahoo.co.uk;*

2014 to May 2014.
Methodology: Four types of sausages were prepared i.e with nitrite, nisin, nisin+nitrite combination, and one without any preservatives (control). Antibacterial effect of Nisin was investigated by artificial contamination of chicken meat with *Salmonella typhimurium* (ATCC 14028) and *Bacillus subtilis* (ATCC 6633) at two different cell densities (10^2 and 10^8 Colony Forming Units (CFU)/g of meat) followed by treatment with nisin and nitrite. The recovery of bacteria was determined by spread plate method using ground sausages samples at precooked, cooked and frozen status. Data analysis was done using statistical analysis system for windows 9.0 software at 95% significant level.
Results: The precooked sausage batter with added nisin, nitrite and combination (nisin and nitrite) which were contaminated with *S. typhimurium* at the level of 10^2 and 10^8 CFU/g meat cell density showed statistically significant reduction in *S. typhimurium* cell number (88%, 82.8% and 90% for10^2 and 89%, 84%, 91% for the latter) reduction respectively and there is reduction of *B. subtilitis* cells also at both concentrations, having 84%, 81% and 86% reduction at low and 89%, 83% and 90% at high cell density. Cooked as well as frozen treatments exhibited a significance reduction (100%) of average colony counts in preservative added samples (nisin, nitrite and combine – nisin with nitrite) compared to control in sausages contaminated with *S. typhimurium* at both cell densities (10^2 CFU/g meat, 10^8 CFU/g meat). In the samples which had low contamination with *B. subtilis* (10^2 CFU/g meat) showed 89.5%, 84.2%, 90.5% reduction compared to control (without nisin and nitrite) in nisin, Nitrite and combine (nisin + nitrite) respectively and in the samples contaminated with high number of cells showed 89.2%, 88.1%, and 91.9% respectively subsequently to cooking. In frozen samples at both contamination levels, both nisin and nitrite revealed a reduction in bacterial count compared to the untreated control.
Conclusion: It is concluded that nisin has the inhibitory effect against *Salmonella typhimurium* and *Bacillus subtilis* and further it can be concluded that, there is a potential of nisin to replace the antimicrobial property of Nitrite in broiler chicken sausage production.

Keywords: Bacillus subtilis; preservative; Nitrite; Nisin; Salmonella typhimurium; Sausage.

1. INTRODUCTION

Poultry meat is the one of major meat type that contributes to the global meat production and consumption production and the per capita consumption of meat have been increased annually. Meanwhile value added meat products also gained a good market demand globally. With the changes in life pattern and industrialization, meat processing industry has become a vital juncture in the world. Thus, processed meat items are produced to utilize the difference carcasses beneficially. Not only that, there are many other purposes such as, production of value added products, increase the marketability and high demand for processed products, to meet the life style requirements, to get economical and quality benefits by incorporating meat with non meat ingredients, increase the shelf life of the meat items and increase the export market and to compete with import products. Therefore in order to achieve these kinds of requirements, meat processing industry is improving day by day. Value added meat products such as, sausage, meat balls, ham, bacon have gained the high market demands. Among the value added meat products sausages are the most popular processed meat type in the market [1]. It is produced from ground meat combine with other non meat ingredients such as binders, emulsifiers, antioxidants, flavors and preservatives. Meat can be undesirable for human intake either because of the living animal has a disease or because of the spoilage through cross contamination while processing. Spoilage can be happened after the slaughter of animals either by chemical breakdown or especially by the micro-organisms. Disease can make the meat aesthetically unacceptable or more importantly, can lead to transmission of infection to human. As well as, meat contamination can be happened due to microbes by many ways such as contaminated hand, water, ingredients, materials etc. [2]. *Salmonella* spp., *Bacillus subtilis*, *Bacillus cereus, Escherichia coli, Camphylobacter* spp. *Clostridium* spp. *Listeria monocytogenes, Lactococcus* spp. are the most common pathogens involve in meat and meat product's spoilage. Some of these microbes such as *Clostridium botulinum* produce lethal toxins. Also most of them such as *Bacillus* spp. *Clostridium* spp. are produced heat resistance spores [3]. As the meat is a good source for bacteria to grow, preservatives are used for meat processing industry in order to get rid of these harmful organisms. Improving the keeping quality

by reducing or killing food borne pathogenic micro-organisms of raw meat/meat products is the utmost important objective of food technologists and microbiologists. In order to have microbiologically safe products, preservative are used as a compulsory component in the process of value addition. In this context, Sodium nitrite and Potassium nitrite plays a vital role in sausage production as a preservative and it can provide bacteriostatic, antioxidant properties and improve the flavour and colour of the sausage [4]. Some other processing steps such as cooking, smoking and freezing also help in the preservation of meat to some extent but the use of Nitrite is a common practice in the process. Although Nitrite shows the beneficial effect as a preservative, there are some adverse effects on human health. It is because of the production of nitrosamine which is carcinogenic to human [4], [5], [6]. And also its effective antimicrobial ability is efficient if the product's pH is lower than 7 [7]. It is reported that the toxic effect of Nitrite was three to five times greater at pH 6 than pH 7 [7]. According to the view of Daniel and his group [8] smoked or grilled meat was linked to higher risk of stomach cancers in the U.S., In addition, they reported that there is an increased risk of dropping dead from cancer, CVD (Cardio Vascular Diseases) causes with high intake of red and processed meat consumption. Because of these arising health problems more people are willing to accept organic or natural product for their consumption [8]. Thus among the all meat preservation techniques, natural preservation techniques are gaining more demand over chemical preservation methods. Recent food-borne microbial outbreaks are driving a search for innovative ways to inhibit microbial growth in the food while maintaining quality, freshness and safety.

With this burning health issue in the meat processing industry, lot of researchers are discovering alternatives for these chemicals with ill health effects. Nisin is one of such possible chemicals that can be used in the meat processing industry as a preservative alternative to Nitrite. Nisin (E 234) is only a natural antibiotic and there are no reports on the development of antibiotic resistance and the toxins development when used in the food production [9]. It is a polypeptide produced by *Lactococcus lactis* belongs to the serological group N. Metick and Hirsh are the first people who used name nisin in 1947 [6,9]. It (E 234) is authorized for food preservation in the European Union by Directive 95/2/EC on food additives.

Because it is non-toxic, heat stable and does not contribute to off-flavours, nisin is commercially used in a variety of foods including dairy, eggs, vegetables, meat, fish, beverages and cereal-based products to inhibit growths of food borne pathogens [10,11]. Nisin has shown the antibacterial activity against most gram-positive bacteria including *Listeria* and spore formers such as *Clostridium botulinum* [12,13]. Nisin is highly inactivated by digestive enzymes and there is no any evidence regarding any cross-resistance occurring to antibiotic [14,15] and also according to the Styles [16] two cell transformation tests have been performed with nisin that is not having any transforming ability [17,18]. There were only few reports available at global level regarding the use of nisin as a preservative in meat processing industry. As there are some negative effects of Nitrite on human health, researchers are trying to find some alternative compounds as a preservative for the production of sausages. Nisin is the one of most suitable chemical compound which they have found [6] and it is a natural lantibiotic which is extracted from polypeptide bacteria. Therefore, according to the literature there is no evidence to prove the negative health effect of nisin for the human consumption [18]. Hence the aim of this study was to investigate the antibacterial effect of nisin (E234) against *Salmonella typhimurium* and *Bacillus subtilis* in the production of chicken sausage.

2. MATERIALS AND METHODS

The study was conducted to determine the possibility of using nisin (E234) as an alternative to Potassium Nitrite in the production of sausage. The antibacterial effect of nisin was investigated against *Salmonella typhimurium* and *Bacillus subtilis* as a Gram negative and Gram positive spore former respectively by artificial contamination at two cell densities to mimic the low contamination and high level of contamination of meat which used for the production of sausages. Prior to the experiment, in order to test the suitability of nisin as an additive, nisin incorporated sausages were prepared without any artificial contamination with microbes. Quality parameters, shelf life and the consumer preference of sausages prepared with nisin were tested. For the confirmation of product safety for human consumption, physical (Color, Taste, appearance, odor, texture) chemical (pH

and moisture, residual Nitrite) and the biological parameters (presence of *E. coli,* total plate count, enumeration of *Staph. aureus*) were tested in a separate experiment.

2.1 Antibacterial Effect of Nisin and Nitrite on Specific Microbes

Meat was artificially contaminated with specific microbes at two different cell densities. For the detection of antibacterial effect of nitrite and nisin, *S. typhimurium* (ATCC14028) and *B. subtilis* (ATCC6633) were used at 10^2 CFU/g of meat and 10^8 CFU/g of meat of densities. Pure cultures of *S. typhimurium* and *B. subtilis* used were kindly donated by Prof Indrani Karunasagar, Dean, Faculty of Biomedical Sciences, Nitte University, Mangalore, India. Cultures were recovered from the glycerol stock and single colony was used for further study. Colonies were grown in Luria Bertani broth (Himedia, India) for the study and both *S. typhimurium* and *B. subitilis* were incubated overnight at 35C° ±2 and at 28C° ±2 respectively.

2.2 Artificial Contamination of Meat by Specific Micro Organisms

In order to sterilize all the ingredients including meat used for the sausage, production was treated with UV, before preparing the sausage batter and sausage batter was prepared as indicated in (Table 1) and it also was sterilized in UV for another 30 minutes before contaminating the batter artificially with specific microbes. Two concentrations of microbes were determined by the optical density at 600nm using UV spectrophotometer (Thermo scientific, Genesys 10 SUV-V1S) according to McFarland 0.5 turbidity standard. Subsequently, the meat samples (20g for each replicate) were infected with *S. typhimurium* and *B.subtilis* at 10^2 and 10^8 CFU/g meat density.

2.3 Incorporation of Nitrite and Nisin to Sausage Mixture

Nisin (0.2g/kg meat) and Nitrite (2g/kg meat) were used as the preservatives in preparing sausages and prior to the preparation of nitrite and nisin solutions, powder forms of these chemicals were treated with UV for 30 minutes in order to remove the microbes if exists. Nisin and nitrite were added to the batter as indicated concentrations and homogenize the batter to have a uniform distribution of chemicals (nisin and nitrite). Each treatment consists of three replicates. The chemical composition of Nisin (E 234) was 50% pure nisin with sodium chloride. As well as the composition of nitrite source was $NaNO_3$ (5%±0.5) and KNO_3 (4.9%±0.5) with salt (NaCl) as a carrier.

Table 1. Preservatives and their concentrations used for the preparation of experimental sausages

Chemical	Concentration
Nisin	0.2 g/kg meat
Nitrite	2 g/kg meat
Nisin + Nitrite (Combination)	0.1 g of Nisin/kg meat+1 g of Nitrite/kg meat

A portion of the sausage batter with preservatives (nisin, nitrite) and without any preservatives (i.e the control) served as the precooked sample and one set of the sample was cooked (to get core temperature 72C°) and showered, this is known as the cooked sample. Meanwhile, another set of the sample was cooked, showered and frozen at -18C° and it was taken as the frozen sample. These three types of samples (precooked, cooked and frozen) were taken for the enumeration of recovered bacteria. Each treatment contained three replicates and reproducibility was checked in a separate experiment.

2.4 Enumeration of Bacteria

XLD (Xylose Lysine Deoxycholate) agar was selected to enumerate the *Salmonella typhimurium* and LB (Luria Bertani) agar was used for *Bacillus subtilis*. Following the homogenization (by using the stomacher) of samples, serial dilutions were made to enumerate bacteria using spread plate method. Subsequent to the spreading, plates were incubated at 37C° and 28C° respectively for *S. typhimurium* and *B. subtilis.* Number of colonies was counted manually after overnight incubation of plates and bacterial numbers were expressed as CFU/g of meat. The enumeration was done for the precooked, cooked and for the frozen samples separately.

2.5 Data Analysis

Data were analyzed by one way ANOVA, using statistical analysis system for windows 9.0 software. When interaction ($p<0.05$) existed among the treatments, the significant difference among treatments were further investigated using Duncan's new multiple-range test. Data

analysis was done using SAS (9.0) at 95% significant level.

3. RESULTS AND DISCUSSION

3.1 Effect of Nitrite and Nisin on *Salmonella typhimurium* and on *Bacillus subtilis* at Difference Cell Densities (10^2 CFU/g Meat and 10^8 CFU/g meat) in Pre Cooked Sausage Batter

Following the preparation of sausage batter specific microbial count was detected in the sample from all the four treatments (nisin, nitrite, Combine; nisin + nitrite, Control; Without nisin and nitrite).

The sausage batter (precooked) with added nisin, nitrite and combination (nisin and nitrite) which were contaminated with *S. typhimurium* at the level of 10^2 CFU/g meat cell density showed statistically significant reduction in *S. typhimurium* cell number (88%, 82.8% and 90% reduction respectively) as well as batter contaminated with high cell density 10^8 CFU/g meat of *S. typhimurium* also showed reduction in cell number (89%, 84%, 91%) compared to the control (without nisin or nitrite) (Table 2, Fig. 1).

When considering average colony counts in batter (precooked) with added nisin, nitrite, combine (both nisin and nitrite) and control which were contaminated with *S. typhimurium* at the level of 10^2 CFU/g meat cell density there was $3.0*10^2$ CFU/g meat, $4.3*10^2$ CFU/g meat, $2.5*10^2$ CFU/g meat and $2.5*10^3$ CFU/g meat of *S. typhimurium* colonies respectively (Table 2). Whereas the batter (precooked) contaminated with *S. typhimurium* at 10^8 CFU/g meat cell density have shown average colony count of $1.9*10^7$ CFU/g meat, $2.9*10^7$ CFU/g meat, $1.6*10^7$ CFU/g meat and $1.8*10^8$ CFU/g meat in batter with added nisin, nitrite, combine (nitrite and nisin) and control (Without nisin or nitrite) respectively (Table 2, Fig. 2).

Sausage batter (precooked) which added nisin, nitrite and combination (nisin and nitrite) showed a statistically significant reduction in *B. subtilis* in both densities (10^2 CFU/g meat and 10^8 CFU/g meat). In the batter contaminated with *B. subtilitis* at 10^2 CFU/g meat of cell concentration, 84%, 81% and 86% reduction was observed in nisin, nitrite and combine (Nisin with Nitrite) samples compared to the control (without nisin or nitrite) respectively (Table. 2, Fig. 1). Their average colony counts there was $5.1*10^2$ CFU/g meat, $5.9*10^2$ CFU/g meat, $4.4*10^2$ CFU/g meat and $3.1*10^3$ CFU/g meat of *Bacillus subtilis* colony in Nisin, Nitrite, combine and control respectively (Table. 2).

At 10^8 CFU/g meat contaminations, 89%, 83% and 90% reduction were observed respectively compared to the control (without Nisin or Nitrite) (Table. 2, Fig. 2). Average colony counts of $2.1*10^7$ CFU/g meat, $3.0*10^7$ CFU/g meat, $2.0*10^7$ CFU/g meat and $2.0*10^7$ CFU/g meat were observed in samples with Nisin, Nitrite, combine and control (without Nisin and Nitrite) respectively (Table. 2).

3.2 Effect of Nitrite and Nisin on *S. typhimurium* and on *B. subtilis* at Difference cell Densities (10^2 CFU/g meat, 10^8 CFU/g meat) in Cooked Sausage Sample

After cooking, all the treatments have shown a significance reduction of average colony counts in preservative added samples (nisin, nitrite and combine – nisin with nitrite) compared to control (without nisin and nitrite) in sausages contaminated with *S. typhimurium* at both cell densities (10^2 CFU/g meat, 10^8 CFU/g meat). 100% reduction was obtained in all the treatments compared to the control. Control sample showed $9*10^1$ CFU/g meat in the cooked sausage samples which contaminated with S. *typhimurium* at low density (10^2 CFU/g meat) and also, in high cell density (10^8 CFU/g meat) contamination, 100% reduction was shown in the treatments of nisin and combined (Nisin & Nitrite) after cooking at 72C° and there was 99.9% reduction in nitrite added samples (Table 2, Fig. 1, Fig. 2). All treatment samples showed the significant reduction of colony count of S. thypimurium following cooking.

There was a significance reduction of microbes in cooked sausage samples contaminated with *B. subtilis* at both densities (10^2 CFU/g meat, 10^8 CFU/g meat). In the samples which had low contamination with *B. subtilis* (10^2 CFU/g meat) showed 89.5%, 84.2%, 90.5% reduction compared to control (without nisin and nitrite) in nisin, nitrite and combine (nisin + nitrite) respectively. But there is no significance difference among average values of nisin (10 CFU/g meat), Nitrite (15 CFU/g meat), Combine (9 CFU/g meat) compared to Control sample (95 CFU/g meat). Cooked sausage samples with

high amount of microbial contamination with *B. subtilis* (10^8 CFU/g meat) had 89.2%, 88.1%, and 91.9% (nisin, nitrite, Combine – nisin with nitrite) of reduction compared to the Control (without nisin and nitrite) and the colony counts were, $1.665*10^3$CFU/g meat, $1.85*10^3$ CFU/g meat, $1.25*10^3$CFU/g meat, $1.55*10^4$ CFU/g meat respectively (Table 2, Fig. 1, Fig. 2).

3.3 Effect of Nisin and Nitrite on *S. typhimurium* and on *B. subtilis* at Difference Cell Densities (10^2 CFU/g meat, 10^8 CFU/g meat) in Frozen Sausage Sample

Cooked and frozen sausage sample which had both the high and low contamination levels with S. *typhimurium* exhibited 100% reduction of S. *typhimurium* in preservatives added treatments (nisin, nitrite, Combine - nisin + nitrite) compared to the untreated control (without nisin and nitrite) and microbial colonies were absent in all the treatment samples while it was 60 CFU/g meat and $1.0*10^3$ CFU/g meat for control at high and low contaminations levels respectively (Table 2, Fig. 1, Fig. 2). Cooked and frozen sausages sample with low number of contaminants, *B. subtilis* (10^2 CFU/g meat) showed 90.8% reduction in nisin added sample with 6 CFU/g meat of average colony count and there was 84.6% reduction in Nitrite treated samples with the colony count of 10 CFU/g meat. Combine preservative added sample (nisin + nitrite) showed 9 CFU/g meat of average colony count of bacteria with 93.8% reduction compared to control sample (without nisin and nitrite). Control sample contained 65 CFU/g meat of average colony count of *B. subtilis*. Samples contaminated with high cell number (10^8 CFU/g meat) showed 11 CFU/g meat, 19 CFU/g meat and 9 CFU/g meat in nisin, nitrite, nisin+nitrite treated samples respectively. The reduction percentages were 91.2, 84.8 and 92.8% respectively compared to the control sample (125 CFU/g meat) (Table 2, Fig. 1, Fig. 2).

Nisin as an effective preservative in the food industry mainly in the dairy industry has been proven in many studies. In a previous study done by Roberts and Hoover in 1996 [19] with *B. coagulans* found that this organism is sensitive to nisin and moreover with high temperature (increasing the temperature from 25°C to 45°C and 70°C) it could lead to complete inhibition. This study also in line with Roberts and Hoover, *B subtilis* showed a reduction of the bacterial number in cooked samples than the precooked samples. This result was expected as the findings of Beuchat and his group [20] and they revealed that with increasing the nisin concentration, increasing the holding time and also the increasing temperature reduces the number of psychrotrophic enterotoxigenic *B. cereus* in beef gravy. Temperature treatments may promote perturbations in the outer membrane, either at low or high temperatures, favoring the action of bacteriocins like nsin which could lead to the inhibition of Gram negative and Gram-positive bacteria [21,22,23], these findings are inline with the current study where we could see the reduction of bacterial count in cooked samples than the precooked samples. In agreement with the current study, Selim and his colleagues [24] found that the nsin could kill 100% of *E. coli*, *Salmonella indica* and *Staphylococcus aureus* after 12, 2 and 4 hours respectively.

According to previous studies by Linda [25] nisin has shown an antibacterial effect against Gram-positive bacteria. With the chelating agents the antibacterial effect has broadened its action against gram- negatives also [26,27,28]. In line with these findings in this study also there was a highest reduction of *Salmonella typhimurium* when use nisin in comparison to the control (without nisin and Nitrite) sample. Nitrite also has shown the effect against bacteria according to the findings of [29,30] According to the view of Rice and pierson, [31] 156 µg/g inhibits the growth of *Salmonella* in frankfurter sausage. The study also exhibited the antibacterial activity of Nitrite even though it is lesser than the nisin. This study accordance with previous findings showed that the nisin and nitrite have good antibacterial effect at low (10^2 CFU/g meat) and higher (10^8 CFU/g meat) contamination levels.

Though the nisin is reported to be highly effective against Gram-positive bacteria without the use of lantibiotics (Mainly nisin) against gram-negative bacteria has also been reported, it depends on the destabilization of the outer membrane by chelating agents, such as ethylenediaminetetraacetic acid (EDTA), by treatment with essential oils or by physical treatments such as freezing, heating or high pressure processing [32,33,34]. A possible explanation for the reduction of bacterial numbers specially the Gram negatives can be due to the cell wall damage either by osmotic (due to the presence of salt) or thermal shock or combine effect, allowing penetrating the nisin to the bacterial cell membrane thereby damaging

the cells. In the current study in agreement with the previous studies, the use of lower temperatures (freezing) and higher temperature (heating) could lead to destabilizing the outer membrane making them sensitive to nisin, leading to lower count of bacteria in cooked and frozen sausage samples compared to the precooked samples.

The pH of the sausages that were prepared was ranging between 5.9-6.0, and this also supported the inhibitory effect on bacteria used and this is in agreement with the study done by Kalchayanand [35] and his group showed acid-stressed, Gram-negative cells of *Yersinia enterocolitica* and *P. aeruginosa* were susceptible to Nisin and pediocin AcH.

Not the nisin alone was responsible for reducing the bacterial count of the sausage but also the other factors during the process of sausage production probably contributed to the death rate acting synergistically to enhance the anti-bacterial properties of the nisin [36,37,38,39].

This study showed that though there is as inhibition of *B. subtilitis* in the treatments it did not destroy 100%. This is in line with the study of Carthy, 1969 [40] and they have shown that the *Bacillus subtilis* endonuclease is stable at 100C° for 30 min and spores are activated with the heat shock. This may be the reason why that *Bacillus subtilis* cells were retained even after cooking.

The inhibition of Clostridium botulinum growth and toxin production is an especially important antimicrobial property of nitrite. Nitrite inhibition of bacteria other than the clostridia has also been reported such as Achromobacter, Aerobacter, Escherichia. Flavobacterium, Micrococcus, and Pseudomonas etc [41]. In accordance with the previous studies, this study also has shown the inhibitory effect of Nitrite on both the bacteria used (S. *typhimurium and* on *B. subtilis*).

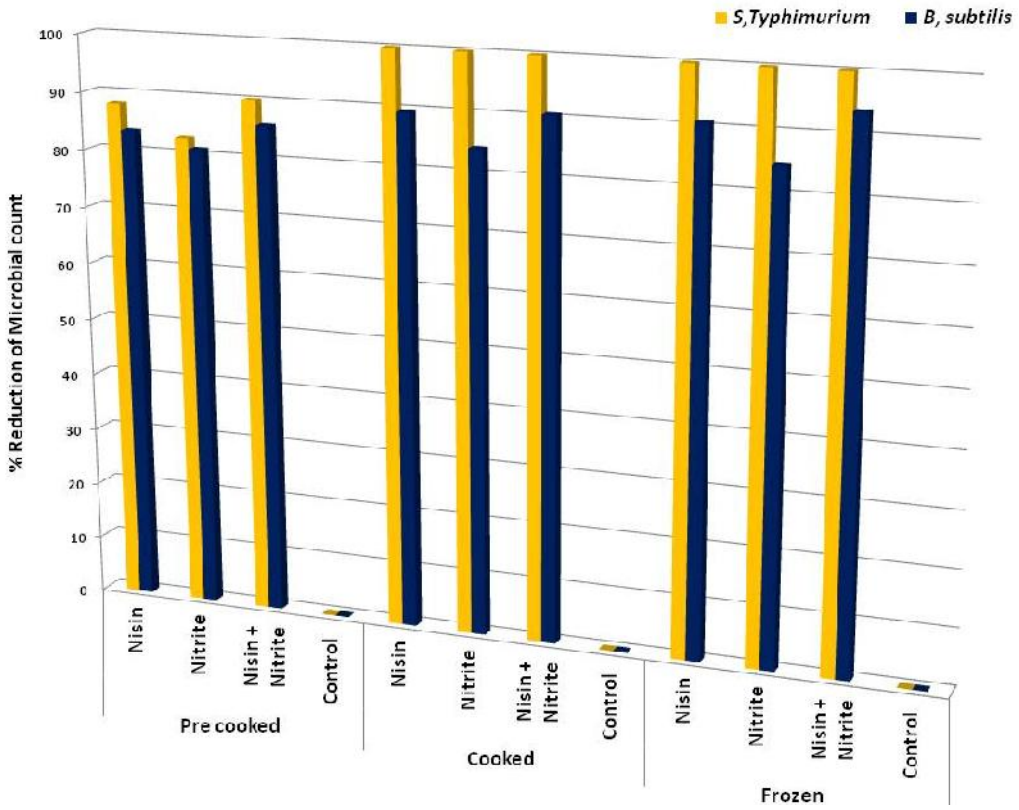

Fig. 1. Effect of Nitrite and Nisin on *S. typhimurium* and *B. subtilis* at difference cell densities (10^2 CFU/g Meat) in pre cooked, cooked and frozen sausage mixture

Table 2. Effect of nisin and nitrite on gram-negative (*Salmonella typhimurium*) and gram-positive (*Bacillus subtilis*) bacteria at two different contamination levels ($10^2$and 10^8 cfu/ g of meat) in the production of chicken sausages at three different stages (precooked, cooked and frozen)

Precooked samples	***S. typhimurium***				***B. subtilis***			
	Nisin	**Nitrite**	**Nisin + Nitrite**	**Control**	**Nisin**	**Nitrite**	**Nisin + Nitrite**	**Control**
	cfu/ g of meat	**cfu/ g of meat**	**cfu/ g of meat**	**cfu/ g of meat**	**cfu/ g of meat**	**cfu/ g of meat**	**cfu/ g of meat**	**cfu/ g of meat**
Contamination of meat with 10^2 CFU/g Meat	$3.0*10^2±20^a$ (88%)	$4.3*10^2±35^a$ (82,8%)	$2.5*10^2±20^a$ (90%)	$2.5*10^3±56^b$ (0%)	$5.1*10^2±35^a$ (83,5%)	$5.9*10^2±42^a$ (81%)	$4.4*10^2±25^a$ (85,8%)	$3.1*10^3±52^b$ (0%)
Contamination of meat with 10^8 CFU/g Meat	$1.9*10^7±1200^a$ (89.4%)	$2.9*10^7±1100^a$ (84%)	$1.6*10^7±1452^a$ (91.1%)	$1.8*10^8±15830^b$ (0%)	$2.1*10^7±2536^a$ (89%)	$3.2*10^7±1542^a$ (83.2%)	$1.9*10^7±5230^a$ (90%)	$2*10^8±12040^b$ (0%)
Cooked samples	***S. typhimurium***				***B. subtilis***			
Contamination of meat with 10^2 CFU/g Meat	0 (100%)	0 (100%)	0 (100%)	90 (0%)	$10±2^a$ (89.5%)	$15±1^a$ (84.2%)	$9±3^a$ (90.5%)	$95±5^b$ (0%)
Contamination of meat with 10^8 CFU/g Meat	0 100%	25±5 99.8%	0 100%	$1.5*10^4±125$ 0%	$1.665*10^3±150^a$ 89.3%	$1.85*10^3±125^a$ 88.1%	$1.25*10^3±182^a$ 91.9%	$1.55*10^4±180^b$ 0%
Frozen samples	***S. typhimurium***				***B. subtilis***			
Contamination of meat with 10^2 CFU/g Meat	0 (100%)	0 (100%)	0 (100%)	60 (0%)	$6±2^a$ (90.8%)	$10±2^a$ (84.6%)	$4±4^a$ (93.8%)	$65±6^b$ (0%)
Contamination of meat with 10^8 CFU/g Meat	0 (100%)	0 (100%)	0 (100%)	$1.0*10^3±25$ (0%)	$11±2^a$ (91.2%)	$19±5^a$ (84.8%)	$9±3^a$ (92.8%)	$125±5^b$ (0%)

**different superscripts within the row are statistically significant at 95% confidence level.*

*** Values within bracket indicate the percentage reduction of colony count*

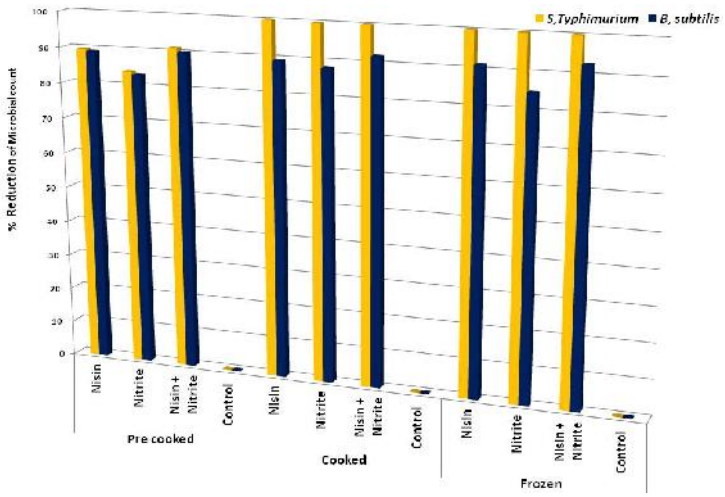

Fig. 2. Effect of Nitrite and Nisin on *S. typhimurium* and *B. subtilis* at difference cell densities (10^8 CFU/g Meat) in pre cooked, cooked and frozen sausage mixture

This study revealed that nisin is having the potency of using in meat processing industry as it had better or more or less similar effect as Nitrite does. Even the pilot study showed that the consumer preference and other quality parameters were up to the acceptable level.

4. CONCLUSION

Nisin has the inhibitory effect against *Salmonella typhimurium* and *Bacillus subtilis* and, there is a potential of nisin to replace the antimicrobial property of Nitrite in broiler chicken sausage production. This study further showed that the synergistic effect of the combination of nisin and nitrite. Therefore it can be stated that *N*isin could be a good alternative to nitrite in the sausage production as a complete replacement or partial replacement.

ACKNOWLEDGMENT

Authors wish to acknowledge Prof Indrani Karunasagar, Dean, Faculty of Biomedical Sciences, Nitte University, Mangalore, India for providing the standard microbial cultures

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kondaiah N. 2004. Value added meat products and development of processed meat sector. Nat Prod Rad. 2004;3(4):281–283.
2. Scannell AGM, Hill C, Buckley DJ, Arendt EK. Determination of the influence of organic acids and nisin on shelf-life and microbiological safety aspects of fresh pork sausage. J Appl Microbiol. 1997;83:407–412.
3. Iulietto MF, Sechi P, Borgogni E, Cenci-Goga BT. Meat spoilage: A critical review of a neglected alteration due to ropy slime producing bacteria. Ital J Anim Sci. 2015;14:3,4011.
DOI: 10.4081/ijas.2015.4011
4. Merino L, Darnerud PO, Toldrá F, Ilbäck NG. Time-dependent depletion of nitrite in pork/beef and chicken meat products and its effect on nitrite intake estimation. Food Addit & Contam Part A, Che Anal Control Expo & Risk Assess. 2016;33(2):186-192.
DOI:10.1080/19440049.2015.1125530
5. Suomi J, Ranta J, Tuominen P, Putkonen T, BäckmanC, Ovaskainen ML, Virtanen SM, Savela K. Quantitative risk assessment on the dietary exposure of Finnish children and adults to nitrite, Food Addit & Contam: Part A. 2016;33(1):41-53, DOI: 10.1080/19440049.2015.1117145
6. Rayman MK, Aris B, Hurst A. Nisin: A possible alternative or adjunct to nitrite in the preservation of meats. Appl. Environ. Microbiol. 1981;41(2):375-380.
7. Essien E. Sausages manufacture principles and practices. 1st ed. wood head publishing limited, Abington Hall, Abington Cambridge CB1 6AH, England; 2003.

8. Daniel CR, Cross AJ, Koebnick C, Sinha R. Trends in meat consumption in the United States. Public Health Nutr. 2011;14(4):575-583. DOI:10.1017/S1368980010002077
9. Gharsallaoui A, Oulahal N, Joly C, Degraeve P. Nisin as a Food Preservative: Part 1: Physicochemical properties, antimicrobial activity, and main uses. Crit Rev Food Sci Nut; 2015. DOI: 10.1080/10408398.2013.76376
10. Bhatia S, Bharti A. Evaluating the antimicrobial activity of Nisin, Lysozyme and Ethylenediaminetetraacetate incorporated in starch based active food packaging film. J Food Sci Technol. 2015;52(6):3504-3512. DOI:10.1007/s13197-014-1414-7
11. Schillinger U, Geisen R, Holzapfel WH. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci Technol. 1996;7:158–164.
12. Ray B. Nisin of *Lactoccocus lactis sub sp. Lactis* as a food preservative. In food bio preservatives of Microbiology organisms, CRC Press, Boca Raton; 1992.
13. Ramu R, Shirahatti PS, Devi AT, Prasad A, Lochana KJ, Zameer MSF, Dhananjaya BL, Prasad MN. Bacteriocins and their applications in food preservation. Crit Rev Food Sci Nut; 2015. DOI: 10.1080/10408398.2015.1020918
14. Tong Z, Zhang Y, Ling J, Ma J, Huang L, Zhang L. An *in vitro* study on the effects of nisin on the antibacterial activities of 18 antibiotics against *Enterococcus faecalis*. PLoS ONE. 2014;9(2):e89209. DOI:10.1371/journal.pone.0089209
15. Hossack DJN, Bird MC, Fowler GG. The effects of nisin on the sensitivity of microorganisms to antibiotics and other chemotherapeutic agents. In antimicrobials and agriculture, ed. M. Woodbine, Butterworths, London. 1984;425-33.
16. Styles JA. A method for detecting carcinogenic organic chemicals using mammalian cells in culture. Br J Cancer. 1977;36(5):558-563.
17. Zhou L, Van Heel AJ, Montalban-Lopez M, Kuipers OP. Potentiating the activity of nisin against *Escherichia coli*. Front Cell Dev Biol. 2016;4:7. DOI:10.3389/fcell.2016.00007
18. Younes M, Aggett P, Aguilar F, Crebelli R, Dusemund B, Filipic M, Frutos MJ, Galtier P, Gundert-Remy U, Kuhnle GG, Lambre C, Leblanc JC, Lillegaard IT, Moldeus P, Mortensen A, Oskarsson A, Stankovic I, Waalkens-Berendsen I, Woutersen RA, Wright M, Herman L, Tobback P, Pizzo F, Smeraldi C, Tard A, Papaioannou A, Gott D. EFSA panel on food additives and nutrient sources added to food (ANS), scientific opinion on the safety of nisin (E 234) as a food additive in the light of new toxicological data and the proposed extension of use. EFSA Journal 2017;15(12):5063,16.
19. Roberts CM and Hoover DG. Sensitivity of Bacillus coagulans spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J Appl Microbiol. 1996;81:363-368. Available:https://doi.org/10.1111/j1365-2672.1996.tb03520.x
20. Beuchat LR, Clavero MRS, Jaquette CB. Effects of nisin and temperature on survival, growth and enterotoxin production characteristics of psychrotrophic *Bacillus cereus* in beef gravy. Appl. Environ. Microbiol. 1997;63:1953–1958.
21. Alakomi HL, Skytta E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl Environ Microbiol. 2000;66(5):2001–2005
22. Boziaris IS, Humpheson L, Adams MR. Effect of nisin on heat injury and inactivation of Salmonella enteritidis PT4. Int J Food Microbiol. 1998;43:7–13.
23. Prudêncio CV, Dos Santos MT, Vanetti MCD. Strategies for the use of bacteriocins in Gram-negative bacteria: Relevance in food microbiology. J Food Sci Technol. 2015;52(9):5408–5417.
24. Selim SA, El Alfy SM, Abdel Aziz MH, Mashait MS, Warrad MF. Evolution of bactericidal activity of selected food additives against food borne microbial pathogens. Biosci. Biotechnol. Res. Asia. 2012;9(1):7-17.
25. Thomas LV, Delves Broughton. Antimicrobials in food - Nisin. 3rd ed. Boca Raton, CRC Press. 2005;237-274.
26. Stevens KA, Sheldon BW, Klapes NA, Klaenhammer TR. Nisin treatment for inactivation of Salmonella species and other gram-negative bacteria. J Appl Environ Microbiol. 1991;57(12):3613-3615.
27. Montiel R, Martín-Cabrejas I, Medina M. Natural antimicrobials and high-pressure treatments on the inactivation of

Salmonella enteritidis and *Escherichia coli* O157:H7 in cold-smoked salmon. J Sci Food Agric. 2016;96(7):2573-8.

28. Jofré A, Garriga M, Aymerich T. Inhibition of *Salmonella* sp. *Listeria monocytogenes* and *Staphylococcus aureus* in cooked ham by combining antimicrobials, high hydrostatic pressure and refrigeration. Meat Sci. 2008;78(1-2):53-59.
29. Lin L, Yun Hu J, Wu Y, Chen M, Ou J, Ling Yan W. Assessment of the inhibitory effects of sodium nitrite, nisin, potassium sorbate, and sodium lactate on Staphylococcus aureus growth and staphylococcal enterotoxin, A production in cooked pork sausage using a predictive growth model, Food Sci. Hum. Wellness; 2017. Available:https://doi.org/10.1016/j.fshw.2017.12.003
30. Cammack R, Joannou CL, Yuan Cui X, Martinez C T, Maraj S R, Hughes M N. Nitrite and nitrosyl compounds in food preservation Biochimica et Biophysica Acta. 1999;475-488.
31. Rice KM, Pierson MD. Inhibition of *Salmonella* by sodium nitrite *and* potassium sorbate in frankfurters. J. Food Sci. 1982;47:1615–1617.
32. Belfiore C, Castellano P, Vignolo G. Reduction of *Escherichia coli* population following treatment with bacteriocins from lactic acid bacteria and chelators. Food Microbiol. 2007;24:223–229.
33. Govaris A, Solomakos N, Pexara A, Chatzopoulou PS. The antimicrobial effect of oregano essential oil, nisin and their combination against Salmonella Enteritidis in minced sheep meat during refrigerated storage. Int J Food Microbiol 2010;137:175–180.
34. Lee J, Kaletunc G. Inactivation of *Salmonella Enteritidis* strains by combination of high hydrostatic pressure and nisin. Int. J Food Microbiol. 2010;140:49–56.
35. Kalchayanand N, Hanlin MB, Ray B. Sublethal injury makes gram-negative and resistant gram-positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. Lett Appl Microbiol. 1992;15:239–243.
36. Raju CV, Shamasundar BA, Udupa KS. The use of nisin as a preservative in fish sausage stored at ambient (28±2ºC) and refrigerated (6±2ºC) temperatures. International Journal of Food Science and Technology. 2003;38:171-185.
37. Scannell AGM Hill C, Buckley DJ, Arendt EK. Determination of the influence of organic acids and nisin on shelf-life and microbiological safety aspects of fresh pork sausage. Journal of Applied Microbiology. 1997;83:407–412.
38. Ilhak OI, Guran HS. Combined antimicrobial effect of thymol and sodium lactate against *listeria monocytogenes* and *Salmonella typhimurium* in Fish Patty. Journal of Food Safety. 2014;34(3):211-217.
39. Ettayebi K, El Yamani J, Rossi-Hassani B. Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. FEMS Microbiology Letters. 2000;183:191-195.
40. McCarthy C, Nester EW. Heat-activated Endonuclease in *Bacillus subtilis*. Journal of Bacteriology. 1969;97(3):1426-1430.
41. Pierson MD, Smoot LA, Robach MC. Nitrite, nitrite alternatives, and the control of *Clostridium botulinum* in cured meats. CRC Critical Reviews in Food Science and Nutrition. 1983;17(2):141-187.

Peer-review history:
The peer review history for this paper can be accessed here:
http://prh.sdiarticle3.com/review-history/24861