Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 4, 626-631

Nitrite free fresh sausage formulated with innovative nitrite alternatives and their impacts on shelf life and quality attributes during refrigerated storage

Essraa A. Youssef[1*], Hemmat M. Ibrahim[1], Mohamed A. Hassan[1], Hamdy A. Zahran[2], Rasha Elsabagh[1]

1Food Hygiene and Control Department, Faculty of Veterinary Medicine, Benha University, Egypt.
2Oils and Fats Department, Food Industries and Nutrition Research Institute, National Research Centre (NRC), 12622 Dokki, Cairo, Egypt.

ARTICLE INFO

Recieved: 18 January 2024

Accepted: 23 February 2024

***Correspondence:**

Corresponding author: Essraa A. Youssef
E-mail address: israa.abdelfatah@fvtm.bu.edu.eg

Keywords:

Nitrite substitute
Nisin
ZON
Hibiscus
Chitosan
Fresh sausage

ABSTRACT

Nitrite is a vital preservative widely used in meat processing. Carcinogenic N-nitroso compounds discovered in processed meat products became serious impacts negatively affect meat industry. The present study provided a nitrite free fresh sausage with a novel method to substitute nitrite with a mixture consisted of (400 ppm nisin+25 ppm Zinc oxide nanoparticles+ 1% *Hibiscus sabdariffa* extract +1% chitosan) employing nisin (N) and Zinc oxide nanoparticles (ZON) as antimicrobials, chitosan (C) as antioxidant and *H. sabdariffa* extract (H) for color improving. *In vitro* antibacterial activity of nisin and ZON against *Clostridium perfringens* reference strain was estimated using agar well diffusion test. *H. sabdariffa* extract was evaluated for its phenolic and flavonoid contents as well as its phytochemical profile was analyzed using HPLC. Four variants of fresh sausage with different treatments were prepared: Nitrite (NT) treated, (N.C.H), (ZON.C.H) and (N.ZON.C.H), packed in polyethylene bags and kept in a refrigerator at 4 ˚C. Samples were examined to assess their pH, microbiological evaluation (aerobic plate, Enterobacteriaceae, yeast and mold counts), also, sensory assessment and shelf life were evaluated. Results exhibited that nisin and ZON have potent antibacterial activity against Cl. perfringens with inhibition zone of 30 mm and 15 mm compared to 30 mm of sodium nitrite. *Hibiscus* extract manifested a high phenolic content about 27.213 mg Gallic acid /g, 2.896 mg QE/g for flavonoids and about 19 main phenolic compounds were detected by HPLC. Results exhibited that N.ZON.C.H group samples showed the lowest pH values, APC, Enterobacteriaceae, yeast and mold counts among sausage variants. Regarding shelf life and sensory evaluation, as expected, N.ZON.C.H showed the highest sensory scores and shelf life (18 days) followed by N.C.H and ZON.C.H that remained accepted till the 15th and finally NT till the 12th day of cold storage. Results suggested that combination of nisin, ZON, chitosan and *H. sabdariffa* extract would be a promising new strategy to replace sodium nitrite and improve safety and quality of fresh sausage.

Introduction

For a long time, food industry has utilized various chemical preservatives to impede the growth of different microorganisms causing food deterioration (El-Saber Batiha *et al*., 2021). In parallel, safe and good quality meat products became highly required especially after clean label concept (Inguglia *et al*., 2023). Nitrite and Nitrate salts (E 249, E 250, E 251 and E 252) for potassium nitrite, sodium nitrite, sodium nitrate and potassium nitrate, respectively, are itemized as preservatives in accordance with food policy (Regulation 1333/2008), these preservatives are usually added into meat batter during processing to obtain the favorable bright red coloration of the products (Karwowska & Kononiuk, 2020).

Nitrite does not impart a pink color for meat products directly, instead, it fixes hemoglobin and myoglobin pigment color. Nitric oxide (NO) which is engendered from nitrite reacts with myoglobin and hemoglobin and form nitro-myoglobin and nitro-hemoglobin which are red in color (Huang *et al*., 2020). Nitrite has an antioxidant activity against lipid oxidation (Dutra *et al*., 2017) through the action of NO that can breakdown the radical chains involved in lipid oxidation (Ji *et al*., 2020) and antimicrobial action against spoilage and pathogenic microorganisms as Clostridium botulinum (Jo *et al*., 2020) and Cl. perfringens (Redondo-Solano *et al*., 2013). Nitrite can play as a nitrosating agent to form nitroso compounds. Numerous epidemiological studies have confirmed a potential relationship between nitrite consumption, N-nitroso compounds formation and possibility of cancer development (Alexander & Cushing, 2011). Moreover, consumption of meat and other foods containing nitrite in their formulation has been reported to increase the risk of colon cancer by World Health Organization (WHO) in 2015 (Perea-Sanz *et al*., 2018).

Consequently, many studies seriously search for other strategies that could be applied as nitrite substitutes in meat products. A significant interest toward natural alternative compounds is preferred as they are comparatively healthier.

Nisin which is a bacteriocin (E234) produced through fermentation of Lactococcus lactis formed from polycyclic antibacterial peptides (about 34-amino acids) (Małaczewska & Kaczorek-Łukowska, 2021) , has a broad spectrum antimicrobial effects against Gram positive and Gram negative bacteria, displays its antimicrobial action through membrane function and permeability period impairment (Roshanak *et al*., 2020) and accepted for human use by FDA in 1988 and by WHO in 1969 (Özel *et al*., 2018). Common problems associated with direct addition of nisin to food are mostly fat adsorption resulting in activity loss, inactivation by proteolytic enzymes and dissimilar distribution food matrix (Gharsallaoui *et al*., 2016). So, one of the most efficient methods to increase the antimicrobial activity of nisin is to combine it with further antimicrobial agents.

Nanotechnology is considered as a rapid growing field in materials of modern science. Antibacterial potential of zinc oxide nanoparticles has got a significant interest globally (Sirelkhatim *et al*., 2015), it has been established against Gram-negative bacteria as E. coli besides Gram-positive bacteria as S. aureus comprising its antimicrobial-resistant variants (MRSA), B. subtilis and vancomycin-resistant enterococci. (Eskandari *et al*., 2023).

Chitosan, a biopolymer that have many reactive functional groups can be chemically modified (Moreno-Vásquez *et al*., 2017). Numerous forms of chitosan bioactivity as antifungal, antioxidant and antimicrobial had been reported (Choi *et al*., 2016), so, it could be applied in meat industry as a bio- preservative.

Plants are remarkable sources for natural pigments with coloring potentials. Roselle (*Hibiscus sabdariffa*) is an annual medicinal plant used

ISSN: 2090-6277/2090-6269/

globally by pharmaceutical and food industries (Da-Costa-Rocha *et al*., 2014). Polyphenols originating from the calyces display antimicrobial action, some of them have also been revealed to exhibit antioxidant activity (Higginbotham *et al*., 2014). Anthocyanins are accountable for the distinctive red color of the calyces of *H. sabdariffa* and can be utilized as natural colorants in many industrial sectors (Shruthi *et al*., 2016).

Taking into account whole the above-mentioned benefits of nisin (N), zinc oxide nanoparticles (ZON), chitosan (C) and *Hibiscus* (H), the current study aimed to evaluate the effects of different nitrite alternatives mixture incorporating 400 ppm N, 25 ppm ZON, 1% C and 1% H extract on physicochemical, microbiological, sensory and shelf life characteristics of nitrite free fresh sausage stored at 4˚C.

Materials and methods

Ethical approval

The present investigation was directed in accordance with perfect clinical practice principles, accepted via members of Animal Experiment Ethical Committee and got Approval no. BUFVTM 10-04-2023 at the Faculty of Veterinary Medicine, Benha University, Egypt.

Material

Chitosan of molecular weight ~ 340, deacetylation degree 85% and moisture content less than 10% was purchased from Marine Hydrocolloids Co, Meron, India. Nisin with 2.5% purity (1×10^6 IU/g) was provided by Sigma-Aldrich (St. Louis, Mo., U.S.A.).

Preparation and characterization of Zinc oxide nanoparticles

Zinc oxide nanoparticles (ZON) was prepared using hydrothermal method according to technique showed by Bulcha *et al*. (2021) applying ($Zn(NO_3)2{\cdot}6H_2O$ and NaOH) as precursors. Morphological evaluation of the prepared ZON was performed by Transmission Electron Microscopy (TEM) (JEM-1200 EXII, JEOL, Japan).

In vitro antibacterial testing assay

The ability of nisin and ZON to inhibit a reference strain of *Clostridium perfringens* ATCC (12915) obtained from the National Institute of Research (Dokki, Giza, Egypt) was estimated using Agar well diffusion test.

Using Milli-Q H_2O, stock solutions of nisin and sodium nitrite at concentration of 0.4 and 0.12 mg/ ml were prepared. ZON solution (0.02 mg/ml) was prepared after strongly vortexed for 15 min to prevent aggregation of particles. Antimicrobial solutions were sterilized by filtration through 0.22-µm pores size filter (Zhu *et al*., 2021) . Methodology of the test was performed as described by Schoster *et al*. (2013). Inhibition zones after anerobic incubation (37°C for 24 h) were measured by a ruler.

Preparation of aqueous Hibiscus. sabdariffa extract

Hibiscus sabdariffa fresh calyxes were dehydrated using a rotary air dryer (50°C for 36 h), filled in nylon-bags, sealed and held in stylofoam box. Dried calyxes were washed by distilled water 3 times. The extraction process was implemented as (1:10 g/ml) at 60°C for a period about 60 min. The extract was filtered using cheesecloth bag (Abdelghany *et al*., 2019). *H. sabdariffa* extract was assessed for its total phenolic and flavonoid contents, screened by HPLC and preserved cooled till time of application.

Determination of total phenolic content (TPC)

Determination of TPC of *H. sabdariffa* was performed using Folin-Ciocalteu (FC) method applying Gallic acid as a standard. Absorbance values had been measured at 760 nm by a UV–VIS Spectrophotometer. In triplicate manner, all determinations were performed. The final results were calculated and expressed as Gallic acid equivalents per gm of the sample (mg GAE/ g) (Ghimire *et al*., 2022).

Determination of total flavonoid content (TFC)

Aluminium chloride colorimetric assay was utilized for evaluation of TFC in *H. sabdariffa* extract applying quercetin as a standard. Flavonoid content of *H. sabdariffa* was established and the final results were evaluated and resulted in expression of mg quercetin equivalent for each gram of sample (mg QE/ g) (Barek *et al*., 2015).

HPLC analysis of H. sabdariffa extract

For quantification of phenolic compounds, the extract was analyzed using HPLC under the conditions: Agilent 1260 series, column: Zorbax Eclipse Plus C8 (4.6 mm x 250 mm i.d., 5 µm)

The mobile phase comprised of water (A) and trifluoroacetic acid (0.05%) in acetonitrile (B) applying 0.9 ml/min flow rate. In a linear gradient, mobile phase was programmed: (0 min for 82% A, 0–1 min for 82% A, 1- 11 min for 75% A, 11- 18 min for 60% A, 18-22 min for 82% A and 22-24 min for 82% A). The detector of multi-wavelength was applied at 280 nm, 5 µl of each sample solution were injected and temperature column was kept at 40°C.

Experimental design

Sausage formulation and processing

Fresh beef sausages were prepared in Food Hygiene Laboratory, Faculty of Veterinary Medicine, Benha university, Egypt. according to Gotardo *et al*. (2023) with some alterations. Sausage formulation compromising 75% lean beef meat, 20% beef fat, 2.0% salt, 0.3% onion, 0.1% pepper and 0.1% garlic was used as a base formulation. Firstly, beef meat and fat were mixed well, and the other ingredients were gradually added. Four treatments were produced, namely: according to their treatments as follow:

NT: Sausage recipe treated with 120 ppm sodium nitrite.

N.C.H: Sausage recipe treated with 400 ppm nisin, 1% chitosan and 1% *Hibiscus* extract.

ZON.C.H: Sausage recipe treated with 25 ppm zinc oxide nanoparticles, 1% chitosan and 1% *Hibiscus* extract.

N.ZON.C.H: Sausage recipe treated with 400 ppm nisin, 25 ppm zinc oxide nanoparticles, 1% chitosan and 1% *Hibiscus* extract.

Sausage ingredients were weighed and mixed thoroughly until obtained a homogenous paste that set in a manual sausage maker. Natural mutton casings were used for fresh sausage stuffing. The sausage variants were packed separately in polyethylene plastic bags. The obtaining products were identified and kept at 4°C to assess the effects of nitrite substitutes treatments on shelf-life and quality characteristics of fresh sausage during storage. Three independent replicates were performed.

Assessment of sausage pH

Ten grams of sausage sample were weighed and homogenized in 90 ml of D.W. Values of pH were measured by pH meter model (Bye model 6020, USA). Triplicate readings were taken (Pearson, 1984).

Microbiological evaluation

The microbiological investigation conducted on sausage variants was for aerobic plate, Enterobacteriaecae, yeast and mold counts. Aseptically,

10 g of samples were weighed discarding the outer layer and homogenized with 90 ml of sterile peptone water (0.1% w/v). Serial ten fold dilution for each sample was made by mixing 1 ml of previously prepared dilution with 9 ml of peptone water. A 100-µl of sample dilution was plated into plate count agar in duplicate. Finally, agar plates were inverted and incubated for 24:48h at 35°C (Park *et al*., 2014). For Enterobacteriaceae, sample dilution was plated on violet red bile glucose agar at 37 °C/ 24 h (Lorenzo *et al*., 2014). Yeast and molds on Sabaroud dextrose agar after incubation at 25°C/5 days (Lashgari *et al*., 2020).

Sensory evaluation of sausage

The effects of different nitrite alternatives on sensory attributes of fresh sausages stored at 4 ˚C were evaluated. Twenty panelists (10 males and 10 females) were selected, with prior experience about sensory characteristics of meat products especially sausage. Sausage samples from each variant were cut into cubes (3 mm thickness), individually labeled and randomly introduced to panelists. Color, odor and texture were estimated. Scores ranged from 1 to 10 being 1 for the lowest and 10 for the highest sensory parameters. Overall acceptability scores were acquired by average scores of color, odor and texture (Alirezalu *et al*., 2021). Sensory investigation of sausage variants was employed in three independent estimates.

Statistical analysis

Statistical analysis was performed by Graph Pad Prism 8.0.2. The analysis used Two-way analysis of variance (ANOVA) (Greenhouse & Geisser, 1959) and p <0.01. The statistical analyses were directed to investigate the influence of different treatments and storage time on quality. Post-hoc analysis was applied using Tukey's HSD test to define which treatments were significantly different from one another when two-way ANOVA produced significant results. The data was expressed as mean ± SD of three triplicates.

Results

Characterization of the prepared ZON by TEM

TEM image of zinc oxide nanoparticles (ZON) prepared by hydrothermal method shows the formation of ZON with an apparent hexagonal structure. The size of the particles is in the range from 50-160 nm with an average diameter of 85 nm (Figure 1).

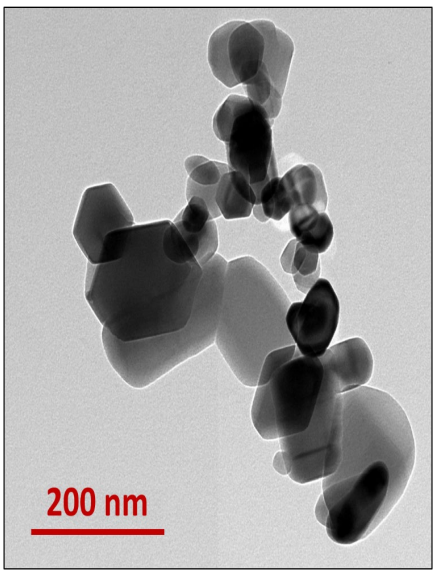

Fig. 1. TEM image of prepared ZON by hydrothermal method depicts hexagonal structure of particles.

In vitro antibacterial testing assay

By applying agar well diffusion test, it was demonstrated that nisin and ZON showed inhibitory activity against Clostridia perfringens. Inhibition zones of 30 and 15 mm were observed compared to 30 mm inhibition zone obtained by sodium nitrite

Total phenolic and flavonoid contents (TPC and TFC) of Hibiscus extract

Phenolic and flavonoid compounds play a considerable role as antibacterial and/ or antioxidants. *H. sabdariffa* extract showed a higher phenolic content than flavonoids. TPC of the extract was 27.213 mg Gallic acid /g while its TFC was 2.896 QE mg/g.

HPLC analysis of H. sabdariffa extract

HPLC confirmed that *H. sabdariffa* extract displayed a high content of phenolic compounds. HPLC identified the main phenolic compounds ~ 19 peaks Figure 2 were recognized and showed the following sequence: Gallic acid > Chlorogenic acid > syrigenic > catechin > rutin> ellagic > methyl gallate > coffeic acid > rosmarinic > diazedin > quercetin > nairngenin > ferulic > hesperetin > coumaric > kaempferol > vanillin > cinnamic acid > pyro catechol. Gallic acid was the prevailing compound among the identified components Table 1.

Table 1. Polyphenolic profiling of *Hibiscus* extract by HPLC.

	Sample			Standard	
	Area	Conc. (µg/ml)	Conc. (µg/g)	Area	Conc. (µg/ml)
Gallic acid	2744.12	241.88	12094.16	226.9	20
Chlorogenic acid	839.53	111.29	5564.34	377.19	50
Catechin	99.28	22.18	1109.13	335.68	75
Methyl gallate	21.24	1.09	54.71	291.16	15
Caffeic acid	13.51	1.07	53.58	226.91	18
Syringic acid	157.41	11.75	587.25	230.52	17.2
Pyro catechol	0	0	0	269.4	40
Rutin	46.68	7.21	360.38	323.79	50
Ellagic acid	23.65	2.43	121.63	583.23	60
Coumaric acid	1.96	0.07	3.58	547.69	20
Vanillin	0.9	0.03	1.7	339.6	12.9
Ferulic acid	4.54	0.27	13.54	335.24	20
Naringenin	5.24	0.49	24.52	320.29	30
Rosmarinic acid	11.42	1.24	62.24	458.62	50
Daidzein	7.61	0.44	22.01	345.53	20
Quercetin	6.54	0.87	43.5	300.84	40
Cinnamic acid	0	0	0	543.25	10
Kaempferol	0.94	0.06	3.03	310.08	20
Hesperetin	3.39	0.17	8.62	393.4	20

pH determination

Values of pH were highly affected by treatments. pH values of the reformulated sausages (N.C.H, ZON.C.H and N. ZON.C.H) were significantly ($p < 0.01$) lower than NT treated samples (Figure 3). N.ZON.C.H group samples had the lowest pH values throughout storage period followed by N.C.H. Results exhibited that NT samples recorded the highest pH values which increased rapidly from 5.83 to 6. 56 after 12 days of cold storage while reformulated sausage samples recorded 6.23, 6.25 and 6.19 for N.C.H, ZON.C.H and N.ZON.C.H, respectively.

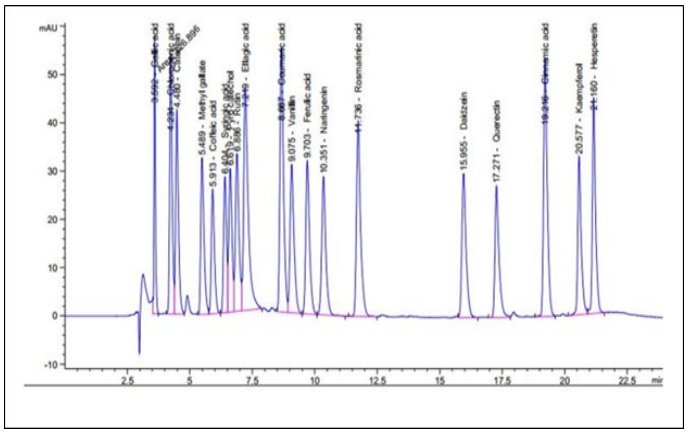

Fig. 2. Chromatogram of phenolic compounds of *H. sabdariffa* extract analyzed by HPLC (19 peaks were identified with their concentration and retention time).

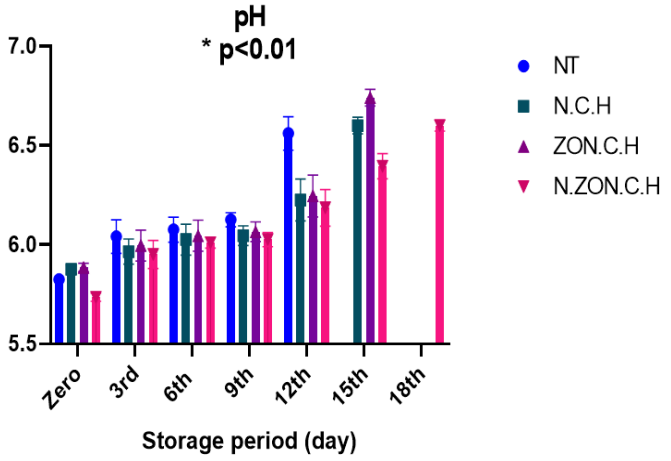

Fig. 3. Changes in pH values of different sausage formulated with nitrite alternatives during cold storage at 4˚C.

Microbiological evaluation

Data in Figure 4 showed the effects of different nitrite alternatives on microbiological quality of fresh sausage during storage period. Aerobic plate, Enterobacteriaceae, yeast and mold counts differ significantly among sausage variants. The best antimicrobial effect was achieved by combination of nisin and ZON in N.ZON.C.H group samples which recorded the lowest bacterial counts ($p < 0.01$).

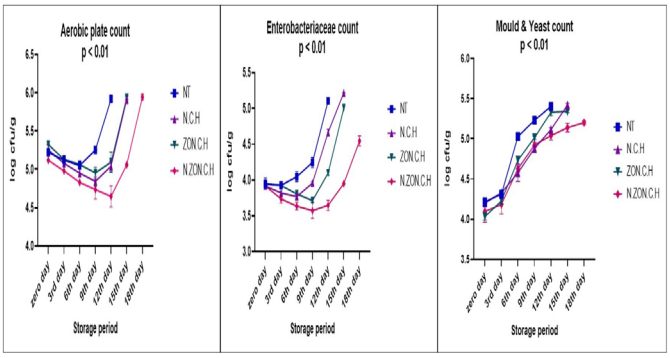

Fig. 4. Effects of nitrite alternatives on microbiological quality of different fresh sausage during cold storage at 4˚C.

APC counts differed significantly between samples, NT samples increased from 5.22 to 5.91 $\log_{10}$ CFU after 12 days of cold storage, 5.25 to 5.91 and 5.32 to 5.93 $\log_{10}$ CFU for N.C.H and ZON.C.H after 15 days while N.ZON.C.H increased from 5.12 to 5.95 $\log_{10}$ CFU after 18 days of cold storage.

Reformulated sausage samples showed lower Enterobacteriaceae counts than NT and the lowest counts were exhibited by N.ZON.C.H group samples. After 12 days of cold storage, Enterobacteriaceae counts increased from 3.95 to 5.11 $\log_{10}$ CFU in NT samples. After 15 days of cold storage, N.C.H counts increased from 3.92 to 5.22 $\log_{10}$ CFU and ZON.C.H increased from 3.94 to 5.01 $\log_{10}$ CFU while, N.ZON. C.H increased from 3.92 to 4.55 $\log_{10}$ CFU after 18 days of cold storage.

As expected, mold and yeast counts were the lowest in N. ZON.C.H that exhibited an increase from 4.09 to 5.20 $\log_{10}$ CFU after 18 days of cold storage. Regarding N.C.H and ZON.C.H increased from 4.21 to 5.40 and from 4.03 to 5.34 $\log_{10}$ CFU after 15 days of cold storage while NT increased from 4.21 to 5.40 $\log_{10}$ CFU after 12 days of cold storage.

Sensory evaluation

Sensory assessment of fresh sausage was significantly differed among sausage variants. Reformulated sausage displayed the highest sensory scores. N.ZON.C.H remained accepted until 18th day of cold storage at 4˚C and recorded the highest scores of overall acceptability based on color, odor and texture assessment among the different variants (Figure 5) followed by ZON.C.H and N.C.H that kept in acceptance till the 15th day then NT till the 12th day of cold storage at 4˚C.

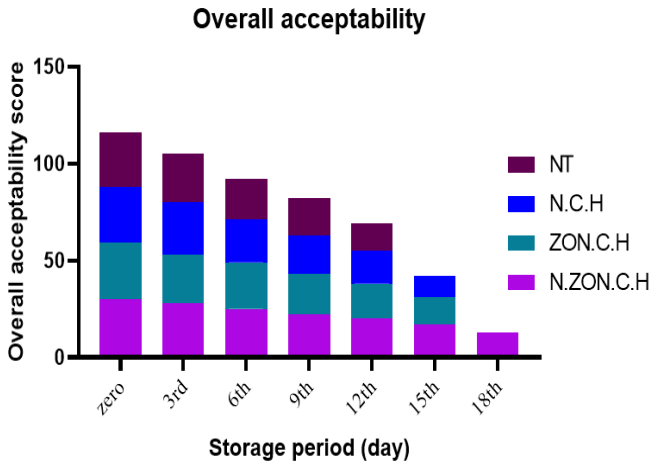

Fig. 5. Effects of nitrite alternatives on sensory attributes of different fresh sausage during cold storage at 4˚C.

Discussion

Risk associated with sodium nitrite addition as a preservative to meat products is a concern for research sector. Therefore, safer substitutes to replace nitrite from meat products formulation is high necessary, the current investigation tried to evaluate the impacts of a nitrite substitute mixture comprised of 400 ppm nisin, 25 ppm ZON, 1% *H. sabdariffa* extract and 1% chitosan.

Clostridial contamination is a highly food safety apprehension as it can produce resistant endospores and potent neurotoxin. A key role to inhibit these potent bacterial strain is nitrite additive, it can inhibit Clostridia through interaction occurs between nitric oxide generated from nitrite and iron sulphur proteins of bacteria leading to suppression of intracellular ATP and pyruvate buildup (Hospital *et al*., 2016). Using agar well diffusion test, *In vitro* antibacterial activities of nisin and ZON against Clostridia were established. Nisin can inhibit vegetative Gram-positive bacteria through formation of pores in cytoplasmic membrane as well as deactivation of cell wall biosynthesis through binding with lipid II (Bierbaum and Sahl, 2009).

Moreover, nisin inhibits germinated bacterial spores outgrowth, it utilizes lipid II as target during outgrowth suppression so, nisin-mediated membrane distraction is necessary for inhibiting development of spores into vegetative cells (Gut *et al*., 2011). Sensitivity of Clostridia to nisin also recorded by Garde *et al*. (2014), their data showed that nisin was efficient against both vegetative cells and spores of the tested Clostridia. perfringens while Sodium nitrite only suppressed spores outgrowth of *C. perfringens* isolate. ZON have an eminent antibacterial potential by rapidly penetrating the cell membrane and liberating Zn^{2+} ions as well as hydrogen peroxide, Zn^{2+} ions can impede active transport and enzymes required for bacterial metabolic activity, damage bacterial cell integrity

through intracellular contents release causing cell dissolution (Seil and Webster, 2012). Susceptibility of Clostridia to ZON (less than 100 nm) found to be consistent with Gomaa *et al*. (2023) and Ibrahim *et al*. (2024).

Hibiscus sabdariffa extract of an attractable red color for its anthocyanin content can be used as a natural colorant to meat products to replace the red color obtained by nitrite. Moreover, results showed that the extract had a high phenolic content about 27.213 mg Gallic acid /g and 2.896 QE mg/g for its flavonoid content. TPC obtained was in range recorded by Zhen *et al*. (2016) and lower than those obtained by Omar *et al*. (2023) who recorded 42.07 ± 0.48 mg GAE/g dry weight of red roselle leaf extract, this diversity may be due to some differences in extraction technique.

Phytochemical screening of *H. sabdariffa* extract using HPLC revealed 19 important compounds as gallic, ferulic and chlorogenic acids, methyl gallate and hespertin. Owing to these compounds, *H. sabdariffa* extract act as potent antimicrobial (Jung *et al*., 2013; Higginbotham *et al*., 2014; Arogbodo *et al*., 2021), antioxidant (Al-Hashimi, 2012) and color improvers of meat products (Santos *et al*., 2022). Data obtained from HPLC profile was in close vicinity to those recorded by Karaaslan (2019).

pH values are substantial because they can significantly influence the microbial balance and eventually affect the shelf life of meat products. The lower values of pH in the reformulated sausage (N.C.H, ZON.C.H and N.ZON.C.H) is mostly due to the antibacterial action of nisin, ZON, phenolic compounds identified in *Hibiscus* extract as well as the antioxidant effect of chitosan that is closely related to its molecular weight (Luo & Wang, 2013). The potent antioxidant activity of chitosan is thought to be through free radical scavenging, metal ion chelation and lipid peroxidation inhibition, moreover, embedding gallic acid (from *Hibiscus* extract active compounds) with chitosan can improve DPPH scavenging activity and reducing power (Abd El-Hack *et al*., 2020), Results interpret that an increase in pH values during cold storage is as a result of decomposition of nitrogenous complexes by microbial or endogenous enzymes (Yuan *et al*., 2016), lower pH values agreed with those recorded by Chang *et al*. (2019) who showed that chitosan can be used for chilled meat preservation. From our obtained results, chitosan could replace the antioxidant activity of nitrite.

Regarding the microbiological quality, the reformulated sausage samples showed lower aerobic plate, Enterobacteriaceae, mold and yeast counts than NT treated samples. Results showed that synergistic antibacterial effect of nisin and ZON could replace the antibacterial function of nitrite. Nisin can inhibit wide range of Gram-positive foodborne bacteria as Streptococcus and Listeria and some Gram negative bacteria as E. coli (Wu *et al*., 2023) while ZON has the potential to suppress many Gram positive and negative pathogens. Similar results were represented by Huang *et al*. (2020) who replaced sodium nitrite with nisin in cured meat and El Asuoty *et al*. (2023) that showed the antibacterial activity of ZON against Gram positive and negative bacteria in refrigerated meat. The lower mold and yeast counts in the reformulated sausage is probably due to the antifungal activity of chitosan, similar results were observed by Arslan and Soyer (2018). Chitosan can bind to cell surface of fungi consequently leading to permeabilization of the plasma membrane and outflow of intracellular constituents, it can also disturb protein biosynthesis and DNA expression through binding to nucleic acid (Meng *et al*., 2020).

Sensory estimation is an efficient technique to get an idea about quality and overall acceptability of meat products (Youssef *et al*., 2021). The current study demonstrated that nitrite substitute mixture could significantly extend shelf life of fresh sausage till the 18th day of cold storage and improve the overall acceptability (based on color, texture and odor). The highly potent antibacterial (nisin and ZON), antioxidant effects (chitosan) could inhibit bacterial growth and protect food matrix from many undesirable interactions which in turn resulted in retardation of spoilage signs and keeping sausage in acceptance score till the 18th day of cold storage, in addition, the desirable red color obtained by *Hibiscus* extract are probably the reasons of higher acceptability in reformulated sausage. Results came in agreement with results obtained by Khorsandi *et al*. (2019) who showed that nisin could extend shelf life of emulsion type sausage, Siripatrawan and Noipha (2012) for chitosan in pork sausage quality, Ayoub *et al*. (2023) for ZON in chicken chilled meat and Pérez-Quintana *et al*. (2019) for *Hibiscus* extract in sausage.

Conclusion

Results demonstrated that nitrite alternative mixture could significantly decrease pH values, improve the microbiological quality and sensory attributes, and extend shelf life of fresh sausage during cold storage at 4°C. Data showed that best results were demonstrated by N.ZON. C.H. The results could provide an innovative method to totally substitute nitrite with nitrite replacers and improve safety and quality of fresh sausage.

Conflict of interest

The authors confirm that there is no conflict of interest.

References

Abd El-Hack, M. E., El-Saadony, M.T., Shafi, M.E., Zabermawi, N.M., Arif, M., Batiha, G.E., Al-Sagheer, A.A., 2020. Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: A review. Int. J. Biol. Macromol. 164, 2726-2744.

Abdelghany, A.M., Menazea, A.A., Ismail, A.M., 2019. Synthesis, characterization and antimicrobial activity of Chitosan/Polyvinyl Alcohol blend doped with *Hibiscus sabdariffa* L. extract. J Mol Struct. 1197, 603-609. doi:https://doi.org/10.1016/j.molstruc.2019.07.089

Al-Hashimi, A.G., 2012. Antioxidant and antibacterial activities of *Hibiscus sabdariffa* L. extracts. African J Food Sci. 6, 506-511.

Alexander, D.D., Cushing, C.A., 2011. Red meat and colorectal cancer: a critical summary of prospective epidemiologic studies. Obes. Rev. 12, e472-e493.

Alirezalu, K., Hesari, J., Yaghoubi, M., Khaneghah, A.M., Alirezalu, A., Pateiro, M., Lorenzo, J.M., 2021.Combined effects of ε-polylysine and ε-polylysine nanoparticles with plant extracts on the shelf life and quality characteristics of nitrite-free frankfurter-type sausages. Meat Sci. 172, 108318.

Arogbodo, J.O., Faluyi, O.B., Igbe, F.O., 2021. *In vitro* antimicrobial activity of ethanolic leaf extracts of *Hibiscus* Asper Hook. F. and *Hibiscus sabdariffa* L. on some pathogenic bacteria. J Sci Res Med Biol Sci. 2, 1-12.

Arslan, B., Soyer, A., 2018. Effects of chitosan as a surface fungus inhibitor on microbiological, physicochemical, oxidative and sensory characteristics of dry fermented sausages. Meat Sci. 145, 107-113. doi:https://doi.org/10.1016/j.meatsci.2018.06.012

Ayoub, A.W., Sayed, S.M., Ammar, M.A., Hefnawy, Y.A., Youssef, A.M., 2023. Novel Edible Bionanocomposites Films Based on Lemon Grass Nanoemulsion and ZnO-NPs for Extending the Shelf Life of Chilled Chicken Meat. Biointerface Res Appl Chem. 13, 573-604.

Barek, M.L., Hasmadi, M., Zaleha, A., Fadzelly, A.M., 2015. Effect of different drying methods on phytochemicals and antioxidant properties of unfermented and fermented teas from Sabah Snake Grass (*Clinacanthus nutans Lind.*) leaves. Int Food Res J. 22, 661.

Bierbaum, G., Sahl, H.-G., 2009. Lantibiotics: mode of action, biosynthesis and bioengineering. Curr Pharm Biotechnol. 10, 2-18.

Bulcha, B., Leta Tesfaye, J., Anatol, D., Shanmugam, R., Dwarampudi, L.P., Nagaprasad, N., Krishnaraj, R., 2021. Synthesis of zinc oxide nanoparticles by hydrothermal methods and spectroscopic investigation of ultraviolet radiation protective properties. J. Nanomater. 2021, 1-10.

Chang, W., Liu, F., Sharif, H.R., Huang, Z., Goff, H.D., Zhong, F., 2019. Preparation of chitosan films by neutralization for improving their preservation effects on chilled meat. Food Hydrocoll. 90, 50-61. doi:https://doi.org/10.1016/j.foodhyd.2018.09.026

Choi, C., Nam, J.-P., Nah, J.-W., 2016. Application of chitosan and chitosan derivatives as biomaterials. J Ind Eng Chem. 33, 1-10.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., Heinrich, M., 2014. *Hibiscus sabdariffa* L.–A phytochemical and pharmacological review. Food Chem. 165, 424-443.

Dutra, M.P., Cardoso, G.P., Fontes, P.R., Silva, D.R.G., Pereira, M.T., Ramos, A.L.S., Ramos, E.M., 2017. Combined effects of gamma radiation doses and sodium nitrite content on the lipid oxidation and color of mortadella. Food Chem. 237, 232-239.

El-Saber Batiha, G., Hussein, D.E., Algammal, A.M., George, T.T., Jeandet, P., Al-Snafi, A.E., Cruz-Martins, N., 2021. Application of natural antimicrobials in food preservation: Recent views. Food Control. 126, 108066. doi:https://doi.org/10.1016/j.foodcont.2021.108066

El Asuoty, M.S., El Tedawy, F.A., Abou-Arab, N.M., 2023. Effect of Antibacterial Activity of Zinc Oxide Nanoparticles against E. coli and Staph. aureus on Quality and Shelf Life of Minced Meat. J. Adv. Vet. Res. 13, 1074-1078.

Eskandari, F., Mofidi, H., Asheghi, B., Mohammadi, F., Gholami, A., 2023. Bringing resistance modulation to methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) strains using a quaternary ammonium compound coupled with zinc oxide nanoparticles. World J Microbiol Biotechnol. 39, 193.

Garde, S., Gómez-Torres, N., Hernández, M., Ávila, M., 2014. Susceptibility of *Clostridium perfringens* to antimicrobials produced by lactic acid bacteria: Reuterin and nisin. Food Control. 44, 22-25. doi:https://doi.org/10.1016/j.foodcont.2014.03.034

Gharsallaoui, A., Joly, C., Oulahal, N., Degraeve, P., 2016. Nisin as a Food Preservative: Part 2: Antimicrobial Polymer Materials Containing Nisin. Crit Rev Food Sci Nutr. 56, 1275-1289. doi:10.1080/10408398.2013.763766

Ghimire, A., Paudel, N., Poudel, R., 2022. Effect of pomegranate peel extract on the storage stability of ground buffalo (*Bubalus bubalis*) meat. Lwt. 154, 112690. doi:https://doi.org/10.1016/j.lwt.2021.112690

Gomaa, N.H., El-Aziz, N.K.A., El-Naenaeey, E.-s.Y., Abdelaziz, W.S., Sewid, A.H., 2023. Antimicrobial potential of myricetin-coated zinc oxide nanocomposite against drug-resistant *Clostridium perfringens*. BMC Microbiol. 23, 79. doi:10.1186/s12866-023-02800-5

Gotardo, L.R.M., Carvalho, F.A.L., Gomes Quirino, D.J., Favaro-Trindade, C.S., Alencar, S.M., Oliveira, A.L., Trindade, M.A., 2023. Study of the Oxidative and Microbiological Stability of Nitrite-Reduced, Vacuum-Packed, Refrigerated Lamb Sausages Supplemented with Red Propolis Extract. Foods. 12, 4419.

Greenhouse, S.W., Geisser, S., 1959. On methods in the analysis of profile data. Psychometrika 24, 95-112.

Gut, I.M., Blanke, S.R., Van Der Donk, W.A., 2011. Mechanism of inhibition of Bacillus anthracis spore outgrowth by the lantibiotic nisin. . ACS Chem Biol. 6, 744-752.

Higginbotham, K.L., Burris, K.P., Zivanovic, S., Davidson, P.M., Stewart, C.N., 2014. Antimicrobial Activity of *Hibiscus sabdariffa* Aqueous Extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a Microbiological Medium and Milk of Various Fat Concentrations. J Food Prot. 77, 262-268. doi:https://doi.org/10.4315/0362-028X.JFP-13-313

Hospital, X.F., Hierro, E., Stringer, S., Fernández, M., 2016. A study on the toxigenesis by *Clostridium botulinum* in nitrate and nitrite-reduced dry fermented sausages. Int J Food Microbiol. 218, 66-70. doi:https://doi.org/10.1016/j.ijfoodmicro.2015.11.009

Huang, L., Zeng, X., Sun, Z., Wu, A., He, J., Dang, Y., Pan, D., 2020. Production of a safe cured meat with low residual nitrite using nitrite substitutes. Meat Sci. 162, 108027.

Ibrahim, R.E., Fouda, M.M., Younis, E.M., Abdelwarith, A.A., Salem, G.A., Elkady, A.A., Rahman, A.N.A., 2024. The anti-bacterial efficacy of zinc oxide nanoparticles synthesized by Nelumbo nucifera leaves against Clostridium perfringes challenge in Oreochromis niloticus. Aquaculture 578, 740030.

Inguglia, E.S., Song, Z., Kerry, J.P., O'Sullivan, M.G., Hamill, R.M., 2023. Addressing Clean Label Trends in Commercial Meat Processing: Strategies, Challenges and Insights from Consumer Perspectives. Foods 12, 2062. Retrieved from https://www.mdpi.com/2304-8158/12/10/2062

Ji, X., Ma, G., Chen, Y., Jin, J., Yu, Q., 2020. Effect of sodium nitrite on fatty acid composition and lipid oxidation of mutton in marinating process. J Gansu Agric Univ. 55, 195-202.

Jo, K., Lee, S., Yong, H.I., Choi, Y.-S., Jung, S., 2020. Nitrite sources for cured meat products. Lwt. 129, 109583. doi:https://doi.org/10.1016/j.lwt.2020.109583

Jung, E., Kim, Y., Joo, N., 2013. Physicochemical properties and antimicrobial activity of Roselle (*Hibiscus sabdariffa* L.). J. Sci. Food Agric. 93, 3769-3776.

Karaaslan, N.M., 2019. A comprehensive study about *Hibiscus sabdariffa* leaves: antioxidant activity, polyphenol profile and macro-and micro-element content. Chem Pap. 73, 791-799.

Karwowska, M., Kononiuk, A., 2020. Nitrates/nitrites in food—Risk for nitrosative stress and benefits. Antioxidants 9, 241.

Khorsandi, A., Eskandari, M.H., Aminlari, M., Shekarforoush, S.S., Golmakani, M.T., 2019. Shelf-life

extension of vacuum packed emulsion-type sausage using combination of natural antimicrobials. Food Control 104, 139-146.
Lashgari, S.S., Noorolahi, Z., Sahari, M.A., Ahmadi Gavlighi, H., 2020. Improvement of oxidative stability and textural properties of fermented sausage via addition of *Pistachio hull* extract. Food Sci Nutr. 8, 2920-2928.
Lorenzo, J.M., Gómez, M., Fonseca, S., 2014. Effect of commercial starter cultures on physicochemical characteristics, microbial counts and free fatty acid composition of dry-cured foal sausage. Food Control 46, 382-389.
Luo, Y., Wang, Q., 2013. Recent advances of chitosan and its derivatives for novel applications in food science. J. Food Process Beverages 1, 1-13.
Małaczewska, J., Kaczorek-Łukowska, E., 2021. Nisin—A lantibiotic with immunomodulatory properties: A review. Peptides 137, 170479.
Meng, D., Garba, B., Ren, Y., Yao, M., Xia, X., Li, M., Wang, Y., 2020. Antifungal activity of chitosan against Aspergillus ochraceus and its possible mechanisms of action. Int. J. Biol. Macromol. 158, 1063-1070. doi:https://doi.org/10.1016/j.ijbiomac.2020.04.213
Moreno-Vásquez, M.J., Valenzuela-Buitimea, E.L., Plascencia-Jatomea, M., Encinas-Encinas, J.C., Rodríguez-Félix, F., Sánchez-Valdes, S., Graciano-Verdugo, A.Z., 2017. Functionalization of chitosan by a free radical reaction: Characterization, antioxidant and antibacterial potential. Carbohydr Polym. 155, 117-127.
Omar, S., Sidik, M.S., Mamat, A., Sanny, M., 2023. Phytochemical analysis, total phenolic content and antioxidant activity of two varieties of *Hibiscus sabdariffa* L. leaves. J. Trop. Resour. Sustain. Sci. 11, 41-48.
Özel, B., Şimşek, Ö., Akçelik, M., Saris, P.E., 2018. Innovative approaches to nisin production. Appl Microbiol Biotechnol. 102, 6299-6307.
Park, M.-S., Wang, J.U.N., Park, J.-H., Forghani, F., Moon, J.-S., Oh, D.-H., 2014.Analysis of Microbiological Contamination in Mixed Pressed Ham and Cooked Sausage in Korea.J Food Prot.77, 412-418. doi:https://doi.org/10.4315/0362-028X.JFP-13-322
Pearson, D., 1984. Chemical analysis of foods., 7th ed.: Churchill Livingstone, USA,PP.168-187
Pérez-Quintana, M., Silva-Chango, J., Caicedo, W., Bravo-Sánchez, L., Arias-Gutiérrez, R., 2019. Use of *Hibiscus* sandariffa extract as a natural antioxidant in sausages. Mol2net. 5, 85-93
Redondo-Solano, M., Valenzuela-Martinez, C., Cassada, D.A., Snow, D.D., Juneja, V.K., Burson, D.E., Thippareddi, H., 2013. Effect of meat ingredients (sodium nitrite and erythorbate) and processing (vacuum storage and packaging atmosphere) on germination and outgrowth of *Clostridium perfringens* spores in ham during abusive cooling. Food Microbiol. 35, 108-115. doi:https://doi.org/10.1016/j.fm.2013.02.008
Roshanak, S., Shahidi, F., Yazdi, F.T., Javadmanesh, A., Movaffagh, J., 2020. Evaluation of antimicrobial activity of Buforin I and Nisin and the synergistic effect of their combination as a novel antimicrobial preservative. J. Food Prot. 83, 2018-2025.
Santos, E.M., Sánchez-Ortega, I., Lorenzo, J.M., Domínguez, R., Munekata, P.E., Falfán-Cortés, R.N., .Rangel-Vargas, E., 2022. Use of *Hibiscus sabdariffa* calyxes in meat products. Front Anim Sci. 3, 876042.
Schoster, A., Kokotovic, B., Permin, A., Pedersen, P.D., Bello, F.D., Guardabassi, L., 2013. *In vitro* inhibition of Clostridium difficile and *Clostridium perfringens* by commercial probiotic strains. Anaerobe 20, 36-41. doi:https://doi.org/10.1016/j.anaerobe.2013.02.006
Seil, J.T., Webster, T.J., 2012. Antimicrobial applications of nanotechnology: methods and literature. Int J Nanomedicine. 7, 2767-2781.
Shruthi, V., Ramachandra, C., Nidoni, U., Hiregoudar, S., Naik, N., Kurubar, A., 2016. Roselle (*Hibiscus sabdariffa* L.) as a source of natural colour: A review. Plant Arch. 16, 515-522.
Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N.H.M., Ann, L.C., Bakhori, S.K.M., Mohamad, D., 2015. Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism. . Nano-micro Lett. 7, 219-242.
Siripatrawan, U., Noipha, S., 2012. Active film from chitosan incorporating green tea extract for shelf life extension of pork sausages. Food Hydrocoll. 27, 102-108.
Wu, M., Ma, Y., Dou, X., Aslam, M.Z., Liu, Y., Xia, X., Hirata, T., 2023. A review of potential antibacterial activities of nisin against *Listeria monocytogenes*: The combined use of nisin shows more advantages than single use. Food Res Int. 164, 112363.
Youssef, E., Saad, S.M., Hassan, M.A., Ibrahim, H.M., 2021. Assessment of the effects of selected plant extracts on quality indices and shelf life of raw chilled chicken meat. Benha Vet Med J. 40, 146-151.
Yuan, G., Lv, H., Tang, W., Zhang, X., Sun, H., 2016. Effect of chitosan coating combined with pomegranate peel extract on the quality of Pacific white shrimp during iced storage. Food Control 59, 818-823.
Zhen, J., Villani, T.S., Guo, Y., Qi, Y., Chin, K., Pan, M.-H., Wu, Q., 2016. Phytochemistry, antioxidant capacity, total phenolic content and anti-inflammatory activity of *Hibiscus sabdariffa* leaves. Food Chem. 190, 673-680. doi:https://doi.org/10.1016/j.foodchem.2015.06.006
Zhu, H., Han, L., Ni, Y., Yu, Z., Wang, D., Zhou, J., He, K., 2021. *In vitro* and In vivo Antibacterial Effects of Nisin Against *Streptococcus suis*. Probiotics Antimicrob Proteins13, 598-610. doi:10.1007/s12602-020-09732-w