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# Exploring A New Synergistic Strategy for Substitution of Sodium Nitrite From Beef Sausage: Impacts on Color, Sensory and Physicochemical Characteristics

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**Abstract**

NITRITE replacement is a global challenge facing meat industry. The current study provides a nitrite substitute to totally replace sodium nitrite from beef sausage formulation. A nitrite replacer comprising 400 ppm nisin (ns), 25 ppm zinc oxide nanoparticles (zno), 1% chitosan (ch) and 1% hibiscus sabdariffa extract (hs) was integrated into beef sausage during manufacturing. Four categories of sausage were prepared as following: nt (120 ppm sodium nitrite), ns/ch/hs, zno/ch/hs, and ns/zno/ch/hs. Chemical quality indices (ph, tbars and tvb-n), instrumental color analysis and sensory evaluation of sausage samples were estimated. The results demonstrated that alternates treated sausage samples exhibited lower ph, tbars and tvb-n values than nt ($p < 0.01$), higher a* redness, better sensory characteristics and the best impacts were attained by ns/zno/ch/hs. In conclusion, ns/zno/ch/hs incorporation into beef sausage formula could be an interesting new strategy to replace sodium nitrite and develop product quality, physicochemical stability, and sensory approval.

**Keywords:** NaNO2; Nisin; ZnO-NP; Chitosan; Hibiscus.

## Introduction

Sodium nitrite (NT) is considered the most common meat additive that plays a major role in enhancing the microbial safety and color of meat products [1]. It is approved to be used as a preservative in meat products such as E249 (Potassium nitrite) and E250 (Sodium nitrite) [2]. Nitrite application has many benefits such as inhibition of microbial growth of most microorganisms especially Clostridium botulinum [3], delaying lipid oxidation as an antioxidant [4], and enhancing aroma and color of cured meat products [5]. Color is considered an important sensory indicator for the quality of meat and meat products [6].The addition of food additives such as nitrite is a common enhancing approach to retain color acceptability and extend the shelf life of meat products [7]. However , this approach leads to the formation of carcinogenic material namely N-nitrosamine that can be formed from nitrite salt after processing of meat products [1], especially under thermal processing of products [8], or due to reaction between nitrite salts and secondary amines [9]. Health risks of nitrite attribute d to its cumulative intake for longterm , it may cause serious dyspnea, vomiting, cyanosis, pulse acceleration, muscle tremors, or even death [10]. Thus, prolonged consumption of nitrite even if it is added within permissible limits,is consider ed unsafe for consumers , and may cause damage to the liver , brain, and kidney [11]. Moreover, toxic N-nitrosamine has a risk of cancer raising as blood, gastric, hepatic, and esophageal cancer. In this regard, processed meat with nitrite is classified by the International Agency for Research of Cancer, as (group 1) carcinogenic material [12]. Consequently , the demand for nitrite-free meat products has increased, and the effort to develop nitrite substitutes for the curing of meat products has intensified [13, 14].

Many safe natural additives have been applied in the meat industry to enhance the quality of meat products and exhibited antibacterial and antioxidant

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activities [15]. Natural bacteriocins such as Nisin (NS, E234) is approved in the meat industry by the FDA as generally recognized as safe additive s (GRAS). It is flavorless, non-toxic, and heat stable [16] ,has antibacterial activity against Gram-positive bacteria such as Clostridium botulinum [17]. NS also, effectively decrease s residual NT in fermented meat products [18]. Another bio-preservative in the meat industry is chitosan (CH) has antioxidant and antimicrobial activity [19]. Moreover, CH combined with radish powder is a promising nitrite alternative to produce NT-free fermented sausages [20]. Nisin nanoparticles mixed with chitosan and plant extracts have proved their ability to replace sodium nitrite, prevent fat oxidation and improve the microbiological quality of nitrite-free frankfurter sausages [21].

One of the novel cutting-edge technologies in the meat industry is nanotechnology [22]. Zinc oxide nanoparticles (ZNO-NP) a GRAS additive [23], showed antibacterial, antioxidant activity and enhancement in the quality of meat and meat products [24, 25]. Additionally, Roselle (Hibiscus sabdariffa) (HS) is a natural food coloring agent and rich in phytochemicals [26]. Its content from hibiscus, anthocyanin, β-carotene and others, give s it a characteristic red color [27]. These additives exhibit one or two nitrite functions. However it sounds be hard to stabilize a nitrite alternate to ensures all roles especially sensory attributes and safety of meat products [13]. Accordingly, synergistic activity between different forms of additives enhances their potential activity [24]. From this point, the aim of this study was to (i) design different nitrite alternates based on synergistic potential role of each NS, CH, and ZNO-NP with HS extract, (ii) evaluate the impacts of nitrite substitution with novel alternates formulations on physicochemical, color, and sensory attributes of chilled raw sausage stored refrigerated at 4°C.

## Material and Methods

*Chemicals*

NS was obtained from Sigma-Aldrich (St. Louis, Mo., U.S.A.), with 2.5% purity, and potency of $10^6$ IU/g. CH was procured from Oxford lab chem., India (De-acetylation 93%, viscosity >75.0%, moisture ≤10.0%, and molecular weight of 161.16).

*Preparation and characterization of ZnO-NP*

ZNO-NP was prepared and identified following the procedure of Bulcha, Leta Tesfaye [28]. A hydrothermal preparation based on Zn $(NO_3)_2 \cdot 6H_2O$ and NaOH precursors was applied. Properties of prepared ZNO-NP were screened by X-ray diffractometer (XRD) (scan range 2 theta, wavelength of $\lambda$ = 1.5406 nm) and Fourier transform infrared spectroscopy (FTIR) (KBr method by a Mattson 5000 spectrometer, Unicam, UK).

*Preparation of fresh watery HS extract*

Watery extract of HS was prepared following the method of Pérez-Báez et al. (2020). Calyxes of HS were obtained from a local market in Cairo, Egypt. They were dried in a convection oven (Digitronic-TFT, Selecta-Group, Barcelona-Spain) at 60 ℃/6 h, and then macerated. Water extract was prepared in 100 mL hot water 87℃/ 10 min, and then cooled at 4 ℃/12 h. Finally, the extract was purified by a Buchner funnel and kept cooled till application.

*Study model*

*Preparation of fresh sausage*

Freshly prepared beef sausage following the recipe of Zhu, Wang [6], with some modifications by using beef meat without fermentation, and stuffed in a natural casing. Beef meat, fat, mutton casings, salt and spices were purchased from a local market in Toukh City , Qalyubia Governorate, Egypt. A base mixture of the fresh sausage was prepared by mixing minced lean beef meat (90%), minced beef fat (10%), sodium chloride (2.5%), glucose (2%), sugar (2%), delta-Glucono-lactone (1%), sodium-sorbate (0.05%), tri-polyphosphate (0.2%), and carrageenan (0.35%). the Prepared base paste was then divided into 4 batches treated as follows:

NT: Sausage paste contained 120 ppm sodium nitrite.

NS/CH/HS: Sausage paste contained 400 ppm NS+ 1% CH+ 1% HS.

ZNO/CH/HS: Sausage paste contained 25 ppm ZNO-NP+ 1% CH+ 1% HS.

NS/ZNO/CH/HS: Sausage paste contained 400 ppm nisin + 25 ppm ZNO-NP+ 1% CH+ 1% HS.

Each patch was separately stuffed into a clean mutton casing. Equal sausage fingers were prepared for each group, packed in polyethylene bags, and kept chilled in the refrigerator at 4°C till spoilage. Samples from each group were collected and examined every 3 days starting from zero-day of preparation. The study was triplicated.

*Chemical quality evaluation of sausage (pH, TBARs and TVB-N)*

Values of pH in collected samples from each group were measured using an electric pH meter (Bye model 6020, USA) in a triplicate manner according to the method of Choi, Song [29]. Thiobarbituric acid reactive substance (TBAR) values were estimated according to the method of Faustman, and Specht [30] , which were equivalents to mg malonaldehyde/ kg of sample. Moreover, Total Volatile Basic Nitrogen (TVB-N) expressed as (mg N/100g of sausage sample), was evaluated following [31].

*Instrumental color evaluation of sausage*

Color analysis was carried out at Cairo University Research Park (CURP), Faculty of Agriculture, Egypt. Colorimeter (Minolta Camera Co, Minolta CR400®, Ltd., Osaka, Japan) was used following comission Internationale de l' Eclairage (CIE Lab system) guidelines [32]. Color grade values were expressed as parameters L* for lightness degree, a* for redness degree, and b* for yellowness degree. Measuring field 8 mm, D65 illuminant, 2° standard observing angle, with calibration to a white standard was applied before measuring. Mean values were calculated based on 6 readings per tray with 2 measurements taken for each sausage slice.

*Sensory evaluation of prepared sausage*

The sensory assessment of sausage samples was implemented in three independent estimations. Sausages were sliced into small sections (1 ± 0.5 cm thickness), and one slice of each processed sample was presented to apanelists group that consisted of (15 males, 35–48 years old and 10 females, 33–42 years old), Food Hygiene and Control Department staff members, familiar with the sensory evaluation of meat products). The sausage samples were randomly presented to panelists and coded by 3-digit random numbers. Each panelist received one slice from each type of fresh sausage variant, denoted as V1, V2, V3 and V4. The panel members were asked to evaluate the sausages based on their color, odor, texture, and overall acceptance, evaluation was applied for each sensory factor [33].

*Statistical Analysis*

Data collected from 3 replicates was statistically analyzed by Graph Pad Prism 8.0.2., using Two-way analysis of variance (ANOVA) with significance $p<0.01$. While instrumental color data was analyzed by one-way ANOVA. Statistical analyses were implemented to evaluate the impact of different nitrite alternatives and storage time on the quality parameters of sausage. Post-hoc analysis was conducted using Tukey's HSD test to determine the significance between groups, when two-way ANOVA produced significant results. Whole values are expressed as mean ± SD of 3 triplicates [34].

## Results

A novel industrial challenge to produce nitrite-free meat products has increased based on the consumer demands to overcome a toxic hazards of nitrite. Based on synergistic capacity, in this study, different mixtures of additives were applied to adjust a novel alternative to nitrite that can replace its functions in meat especially in attractive color of meat products.

*Characterization of ZnO-NP using XRD and FTIR*

The X-ray diffraction pattern of the prepared ZnO nanoparticles is shown in Fig. (1. A). The pattern depicts the appearance of sharp diffraction peaks at $2\theta$ =31.465°, 34.476°, 35.927°, 47.537°, 56.228°, 62.479°, 67.557°, and 68.748° which could be indexed to the crystal planes (100), (002), (101), (102), (110), (103), (200) and (201), respectively, of hexagonal wurtzite structures of ZnO (JCPDS No. 36-1451). The particle size (D) of the prepared ZnO can be estimated from the well-known Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos \theta}$$

Where D is the nanoparticle average diameter, K is Scherrer (≈0.9), $\lambda$ is the X-ray radiation wavelength, $\theta$ is the peak angle in radian, and $\beta$ is the width at half maximum (FHWM) in radian of the respective XRD peak. Here, will use the high intense peak located at $2\theta$= 35.927°.

Fig. 1B depicts the FTIR spectrum of the prepared ZnO nanoparticles. The peak centered at about 1423 could be attributed to Zn $(OH)_2$ while peaks at 875 and 660 $cm^{-1}$ are attributed to Zn-OH bending vibrations. The formation wurtzite phase of the ZnO is confirmed by the appearance of the Zn-O stretching mode at 440 $cm^{-1}$. The disappearance of any higher frequency absorption bands from 4000-200 cm-1 confirms the formation of pure ZnO nanoparticles.

*Chemical Analysis of Sausage samples:*

Impacts of synergistic nitrite alternative formulation on physic-chemical parameters (pH, TBARs and TVB-N values) of fresh sausages during cold storage are presented in Fig (2).

The values of the investigated parameters exhibited a slight increase during the period of cold storage in NT-treated sausage, while alternated formulation (NS/CH/HS, ZNO/CH/HS, and NS/ZNO/CH/HS) significantly ($p < 0.01$) delayed the rate of increase of chemical parameters compared to NT group. The formulation of NS/ZNO/CH/HS exhibited higher impacts on pH, TBARS, and TVB-N.

*Color parameters*

Impact of different nitrite alternates provided with HS extract as a natural coloring was illustrated in Table (1). NS/CH/HS, ZNO/CH/HS, NS/ZNO/CH/HS treated sausage showed higher in redness a* and yellowish b* and lower in lightness L* than those of NT treated sausage. Alternates treated sausage samples exhibited an increase in a* redness probably for high anthocyanin content of hibiscus extract that impart the characteristic red color.

*Sensory evaluation*

Impacts of novel nitrite alternate formulation on fresh sausage, comparatively with nitrite, was illustrated in Fig. 3. Sensory attributes (color, odor, texture, and overall acceptability) were significantly influenced ($p<0.01$) by novel nitrite alternates. the Chilled fresh sausage was accepted till the 12th day of storage in the NT group , While, those of NS/CH/HS, ZNO/CH/HS, and NS/ZNO/CH/HS remained within an accepted range (shelf life) till the 18th day of storage. Sausage with NS/ZNO/CH/HS showed higher acceptability.

## Discussion

ZnO nanoparticles possess broad antibacterial potentials and its activity against different bacterial strain s is reported [35]. The calculated D is □80 nm. Peaks obtained in the XRD pattern of the prepared ZnO nanoparticles found at (31.46, 34.47, 47.53, 56.22 and 62.47) were in close resemblance to peaks shown by Akbar and Anal [35] who estimated the antibacterial action of ZnO in poultry sausage, moreover, XRD peaks were found to be consistent with Hameed, Sharmoukh [36]. Similar FTIR peaks were showed by Hameed, Sharmoukh [36] and Zyoud, Ganesh [37].

Quality characteristics of meat and meat products are highly contingent on lipid and protein the oxidation reactions. Secondary metabolites of oxidation process accumulate in meat products and induce an increase in chemical parameters (TVB-N, pH and TBARS values), which negatively impact sensory attributes and consumer's acceptability [38]

A delay in increasing of TBARs in NS/CH/HS, ZNO/CH/HS and NS/ZNO/CH/HS treated sausage, confirms the antioxidant capacity of examined NT alternates., TBARS monitor s the degree of lipid oxidation [39]. Peroxides break down into ketones and aldehydes leading to auto-oxidation of products, this is evaluated by TBARS [40, 41]. Also, there was a delay in TVB-N in treated nitrite substituted sausage. The increase may be due to the accumulation of amines and ammonia basic compounds, which result from protein breakdown [42, 43]. It was found that no previous studies manipulated the same mixture in this study. But, each parameter of the formulated alternates separately has a potent antioxidant activity leads to enhancement of chemical quality of meat, shelf life time and sensory of meat and meat products, as previously mentioned by Araújo et al., (2018) [57]; their data showed that nisin combined with essential oils was effective in controlling fresh sausage stability, Morsy, Elsabagh [24] ; showed that nisin, ZnO-NPs, EDTA-NPs and lysozyme were effective to improve safety and quality of chilled meat products, AbdEl-Aziz, Ibrahim [44]; clarified the antibacterial potential ZnO in minced meat kept at 4 °C for a period of 17 days, El Asuoty, El Tedawy [45] ; who represented that ZnO NPs were effective for shelf life extension and improving bacteriological quality of minced beef, Fernando, Jo [19] ; showed that chitosan induced positive impacts on shelf life and organoleptic properties of meat products, and Jung and Joo [46] who observed that combination of hibiscus extract and soybean oil were effective to obtain high sensory reception and appropriate physicochemical properties in pork patty. Mixtures of these antioxidants synergistically enhanced their antioxidant capacity.

One of the main roles of nitrite in the food industry is developing the color and aroma of products [47]. Nitrite substitution based on using natural colorants is urgent [48, 49]. Color is a critical parameter in evaluating the quality of meat products and its stability in these products is essential as any alterations can affect their sensory characteristics and consequently consumer acceptance.

Providing formulations of nitrite alternates with HS extract rich with anthocyanin's precursors enhanced the redness of sausage higher than those with NT. Results are consistent with Gibis and Weiss [50] who showed that anthocyanin from hibiscus could improve the color of beef patties. Anthocyanin's precursors enhance the brilliant red color of HS extract [51]. Moreover, its application enhances the safety of products and overcomes the negative impacts of synthetic dyes on human health [52]. From a coloring point, HS extract is a good choice to be an alternative to synthetic food colorants.

One of the indispensable roles of nitrite is the enhancement of sensory attributes. So, the sensory acceptability of sausage with alternative the formulation was taken into consideration in designing formulation of alternates. The results of the sensory evaluation were aligned with those of physic-chemical analysis (PH, TBARS, and TVB-N).

Each parameter of the formulated alternates separately enhances shelf lifetime and sensory of meat and meat products. NS and ZNO-NP synergistically enhanced sensory attributes of meat products [24], and CH also, enhanced the acceptability of different meat products [19]. Moreover, HS extract enhances the color of products [53].

Mixtures from these active compounds synergistically enhanced the acceptability of sausage through their potent antibacterial and antioxidant properties. Nisin is thought to depolarize the cytoplasmic membrane and induce the outflow of cytoplasmic ions of bacterial cells [54], release of antimicrobial ion s is associated with the antibacterial role of ZnO nanoparticles [35]. The antioxidant potential of chitosan is assumed to delay oxidative rancidity by acting as a chelator for the transference of metal ions that contribute to lipid oxidation [55]. Hibiscus, malic, gallic, citric, ferulic, chlorogenic acids, quercetin, kaempferol and hibiscus lactone and other hibiscus phytochemicals act as potent antioxidants through a strong scavenging effect on free radicals and reactive oxygen [56].

## Conclusion

Our research highlighted the efficacy of 400 ppm nisin, 25 ppm zinc oxide nanoparticles, 1% chitosan and 1% Hibiscus sabdariffa extract incorporation into beef sausage formulation to replace sodium nitrite. The results of oxidation stability proved that alternate substances had potent antioxidant capacity in terms of lower pH, TBARs and TVB-N values than nitrite samples. Colorimetric parameters were showed to be significantly linked to Hibiscus additive (anthocyanin) in higher a* redness values. High sensory profile and extended shelf life till the 18th day of cold storage proved the positive impacts of the nitrite substitutes on the product quality. The interesting results shown in our investigation represent a step towards the safety of meat products while enhancing their sustainability. Overall, our attained results demonstrated that NS/ZNO/CH/HS incorporation into beef sausage formula could be an interesting new strategy to replace sodium nitrite and develop product quality mainly concerning its physicochemical stability and sensory approval.

*Conflicts of interest*

The authors have no relevant financial or non-financial interests to disclose.

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*Ethical of approval*

This study was conducted following the principles of perfect clinical practices and confirmed by Animal Experiment Ethical Committee members with Approval no. BUFVTM 10-04-2023 (Faculty of Veterinary Medicine, Benha University, Egypt)

**TABLE 1. Variation in instrumental color screening values of chilled fresh sausages substituted with different nitrite alternates instead of nitrite.**

| Groups | $L^*$ | $a^*$ | $b^*$ |
|---|---|---|---|
| **NT** | 54.54± 0.11$^a$ | 6.19 ± 0.17$^a$ | 14.09 ± 0.38$^a$ |
| **NS/CH/HS** | 49.9 ± 1.01$^b$ | 9.29 ± 0.26$^b$ | 15.11 ± 0.40$^a$ |
| **ZNO/CH/HS** | 50.65± 0.37$^b$ | 6.57 ± 0.35$^a$ | 14.86 ± 0.74$^a$ |
| **NS/ZNO/CH/HS** | 50.36 ± 0.61$^b$ | 9.16 ± 0.13$^b$ | 16.55 ± 0.84$^b$ |

Values expressed as mean ± SD.
Small letter illustrated significant ($p < 0.01$) between different groups.
Grades of color evaluation expressed as L* for lightness, A* for redness, and B* for yellowness

A

B

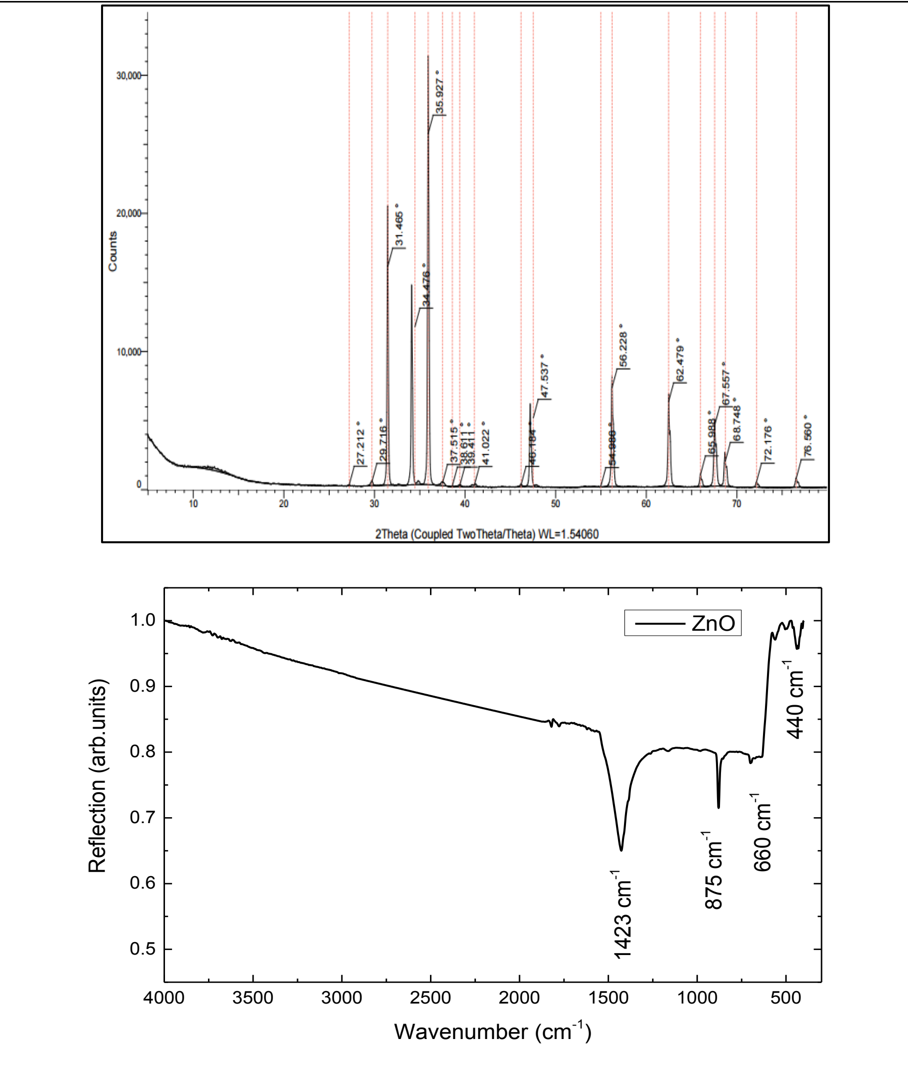


**Fig. 1. A: X-ray diffracto-meter (XRD) (scan range 2 theta, wavelength of λ = 1.54060 nm). B: Fourier transforms infrared spectroscopy (FTIR) screening of prepared ZO-NPS.**

a

b

c

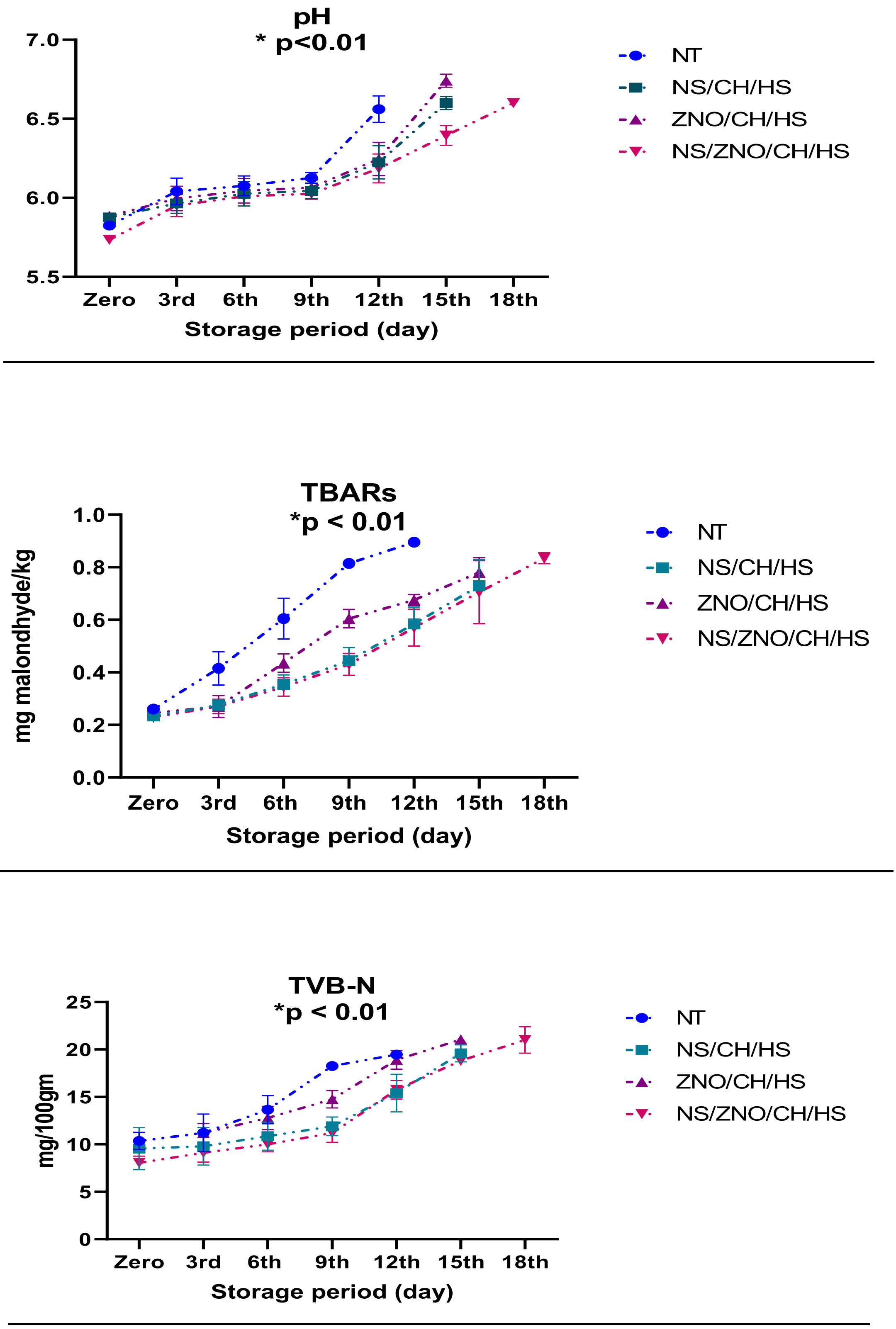


**Fig. 2. Changes in chemical parameters (pH, TBARs and TVB-N) of chilled fresh sausages substituted with different nitrite alternates instead of nitrite.**

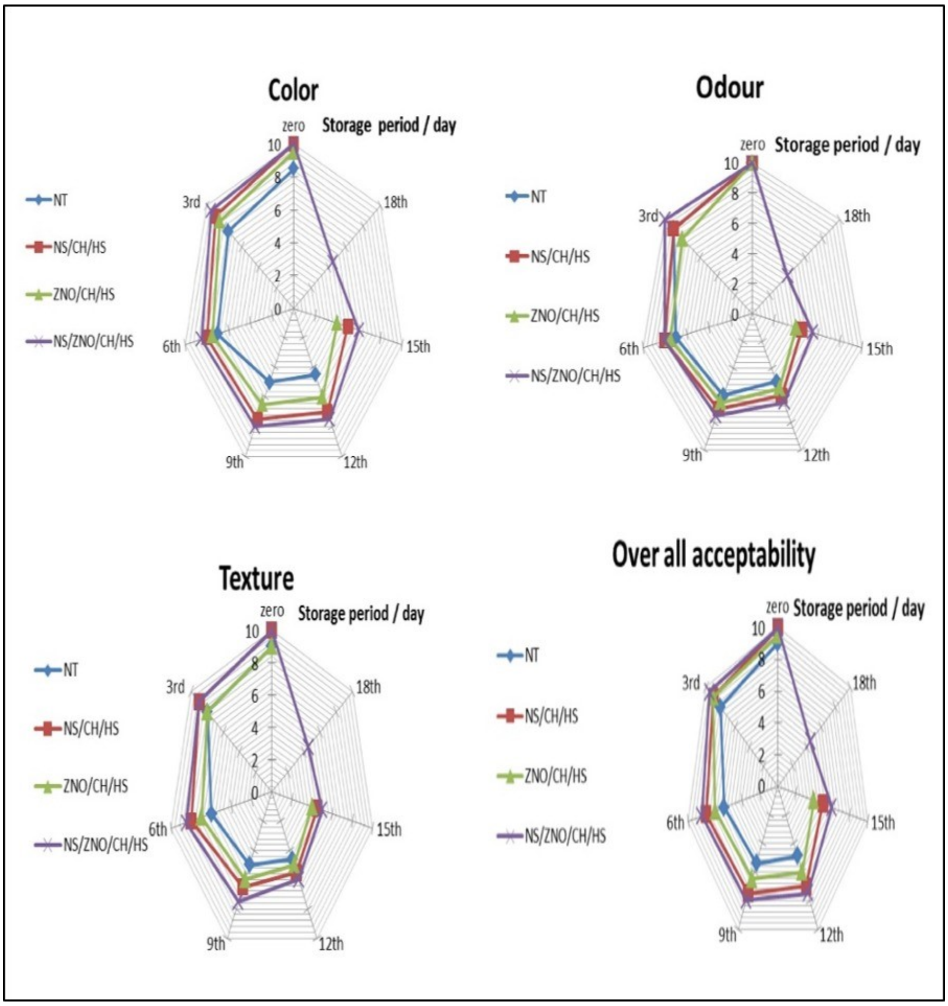


**Fig. 3. Sensory acceptability of chilled fresh sausages substituted with different nitrite alternates instead of nitrite.**

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## استكشاف استراتيجية تآزرية جديدة لاستبدال نيتريت الصوديوم من النقانق البقرية: تأثيراتها على اللون والخصائص الحسية والفيزيائية والكيميائية

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**الملخص**

يعد استبدال النتريت (NT) تحديًا عالميًا يواجه صناعة اللحوم. توفر الدراسة الحالية بديلاً كليا للنيتريت في تركيبة النقانق البقري و يقوم هذا البديل علي دمج (400 جزء في المليون من النيسين NS، و25 جزء في المليون من جزيئات أكسيد الزنك ZNO ، 1% شيتوزان CH و 1% من مستخلص الكركديهHS ). تم تحضير أربع فئات من النقانق على النحو التالي :
(NS/ZNO/CH/HS, ZNO/CH/HS, NS/CH/HS, NT). تم تقييم مؤشرات الجودة الكيميائية (TVB-N، TBARs، pH) ، تحليل الألوان الآلي والتقييم الحسي لعينات النقانق. أظهرت النتائج أن عينات النقانق المعالجة بالبدائل سجلت أقل قيم في مؤشرات الجودة الكيميائية مقارنة بالعينات المعالجة بمادة النيتريت و تميزت بلون أحمرأعلي و خصائص حسية أفضل و أفضلهم تأثيرا كان للبديل NS/ZNO/CH/HS الذي يعتبر دمجه أثناء تصنيع النقانق استراتيجية جديدة لاستبدال مادة النيتريت و تطوير جودة المنتج و استقراره الكيميائي و قبوله الحسي.

**الكلمات الدالة:** النقانق، النيتريت، النيسين، ZnO-NP، الشيتوزان، الكركديه.