

Inhibitory Effect of Blueberry, Curcumin, Prebiotic, and Probiotic on N-Nitrosamine Formation in Cooked Meat Products by Gastrointestinal Simulation

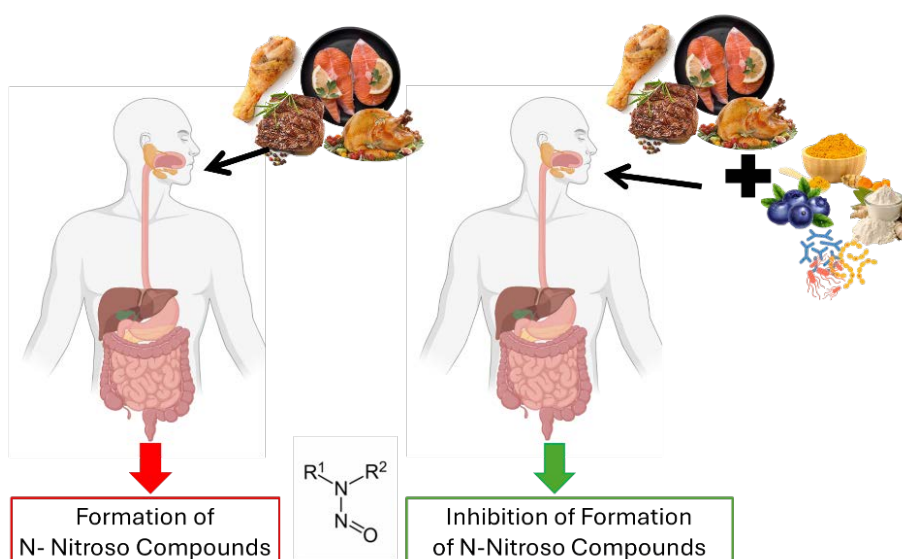
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Received December 10, 2024; Revised January 11, 2025; Accepted February 18, 2025

Abstract Meat has high nutritional content as it contains proteins, vitamins, minerals, and essential fatty acids. However, during the processing and eating of meat products or endogenously, biogenic amines such as N-nitroso compounds are formed. The aim of this study was to determine the N-nitrosamines in unprocessed cooked meat products, to examine the formation of N-nitroso after cooking and in vitro digestion, and to inhibit this formation by adding prebiotic, probiotic, and blueberry and curcumin extracts to meat products. After in vitro digestion, significant increases of 85% in beef, 100% in chicken, 183% in turkey, and 316% in salmon were observed in the nitrosamines of the cooked meat products. In the in vitro digestion of cooked meat products with supplements, the greatest reduction in N-nitrosodimethylamine formation was provided by inulin and probiotic (58%) in chicken and by blueberry extract (47%, 72%, and 68%, respectively) in beef, turkey, and salmon. The greatest reduction in N-nitrosodiethylamine formation was provided by blueberry extract (83%) in beef and by inulin (87%, 78%, and 81%, respectively) in chicken, turkey, and salmon. It is shown that high temperature, the cooking process, and protein, fat, iron, and moisture content affect the possible formation of nitrosamines in meat products. Postdigestive increases in nitrosamines in cooked meat products are associated with gastrointestinal tract conditions. Nitrosamines can be effectively inhibited by the addition of antioxidants, prebiotic, and probiotic added to various meat products. This study may provide guidance for understanding the formation mechanism of nitrosamines in meat products and developing inhibition strategies.



Keywords: beef, chicken, salmon, NDMA, NDEA, supplements, digestion

Cite This Article: Hafsa Sena Sargın Mızrak, and Ayhan Dağ, “Inhibitory Effect of Blueberry, Curcumin, Prebiotic, and Probiotic on N-Nitrosamine Formation in Cooked Meat Products by Gastrointestinal Simulation.” *Journal of Food and Nutrition Research*, vol. 13, no. 2 (2025): 77-86. doi: 10.12691/jfmr-13-2-4.

1. Introduction

Meat is a valuable source of nutrients and an important part of the diet. Consumption of meat and meat products contributes to the intake of many essential nutrients, including protein, essential fatty acids, and various vitamins and trace minerals such as zinc and iron [1]. Meat consumption has increased by 58% in the last 20 years [2]. However, a study conducted by the International Agency for Research on Cancer reported an association between red and processed meat and colorectal cancer. Many compounds found in meat and meat products can be precursors for the formation of N-nitrosamines (NAs) [3]. The most common volatile N-NAs in meat products are N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopiperidine, N-nitrosopyrrolidine, and N-nitrobenzylmorpholine. Among these, NDMA and NDEA are considered the most volatile NAs in terms of oncogenicity and genotoxicity [4].

The European Food Safety Authority (EFSA) Panel on contamination in the food chain, in a study conducted among five food categories, stated that the food category that caused the most NA exposure was “meat and meat products” [5]. According to a World Health Organization report, the acceptable daily intake is 96.0 ng/day for NDMA and 26.5 ng/day for NDEA [6]. However, there is no EU legislation regulating the levels of NAs in foods [7].

N-NAs are formed when nitrosating precursors such as nitrites react with secondary amines derived from protein and lipid oxidation products in meat. NA formation depends mainly on nitrite intake and processing conditions in meat products. This formation is prolonged by storage time, high acidity (pH 2.5–3.5), and high temperature [8]. However, processes such as marination, fermentation, and curing and cooking techniques using temperatures above 130°C such as frying or barbecuing can also increase the formation of NAs [9].

The freshness of raw meat products plays an important role in NA formation because chemical changes during storage can lead to the formation of NAs during processing. Unsaturated fatty acids present in fresh meat products act as nitrite scavengers and thus can inhibit the formation of NAs [10]. As meat loses its freshness, oxidation of adipose tissue also leads to the formation of malondialdehyde, a secondary product of lipid oxidation that can catalyze the formation of NAs [11]. Studies have shown that NDEA formation is increased in stale pork due to the degradation of sarcoplasmic proteins and protein and lipid oxidation [12]. NAs can also be produced endogenously by consuming meat products [9]. In the gastrointestinal tract, NA precursors such as red meat, protein, nitrate, nitrite, and heme iron cause the endogenous formation of S-nitrosothiols (SNOs) and NAs through intestinal or microbiota-mediated pathways and are finally excreted via feces [13].

Approximately 7% of dietary nitrates can be reduced to nitrite by bacterial nitrate reductase in the oral cavity. When nitrite reaches the stomach, it is converted to nitrous acid under low pH conditions and can react with secondary amines and thiol groups obtained from the proteolysis of protein-containing foods, leading to the

formation of NAs and SNOs [13]. SNOs facilitate the formation of NO and nitrosylation of heme iron from meat products in the small intestine [3]. Nitrating precursors formed from NO produced by inducible NO synthase in colonocytes cause further formation of endogenous NAs together with intestinal microbiota [14]. Endogenous conversion of nitrite to NA compounds increases with low antioxidant content of the diet, and this may be an important risk factor for the development of insulin resistance, diabetes mellitus, metabolic diseases, and many types of cancer (oral, esophageal, stomach, gastrointestinal system, etc.) [15].

To increase the nutritional quality of meat products, innovative processing strategies are being investigated to include foods rich in bioactive components (probiotics, antioxidants, and dietary fibers) and to inhibit the formation of harmful compounds (NAs and biogenic amines) during processing [16].

With the classification of nitrite-cured meat as a Group 1 carcinogen by the International Agency for Research on Cancer, vitamins, vegetable extracts, spices, herbs, and fruits are being investigated as alternatives to nitrite and nitrate [17]. Various compounds in the diet, such as vitamins C and E and phenolic and sulfur compounds found in fruits and vegetables, have an inhibitory effect on NA formation [18].

Phytochemicals such as flavonoids and curcuminoids have antioxidant effects [19]. Blueberries contain phenolic compounds such as anthocyanins, flavonoids, stilbenes, tannins, and phenolic acids, indicating that they are one of the richest sources of antioxidants among all fresh fruits and vegetables [20]. In addition, curcumin, the bioactive compound of turmeric, is a polyphenol with strong anti-inflammatory properties and has been the subject of many studies on the prevention and treatment of metabolic diseases in terms of its effects on inflammatory and oxidative balance [21,22]. Phytochemicals can potentially inhibit the formation of NAs when consumed with meat [11].

In addition to the addition of antioxidants for NA inhibition, some bacteria have also shown efficacy in nitrite inhibition and conversion. Lactic acid bacteria (LAB) are used to control potential pathogens and as an inhibitor in food processing and contain specific enzymes that can inhibit NA [23].

The EFSA has granted qualified safety status to many LAB belonging to the genera *Carnobacterium*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Lactobacillus* [24]. These starters produce various compounds with antimicrobial capacity (lactic acid, acetic acid, hydrogen peroxide, and bacteriocins) [25], lowering the pH of the medium and inhibiting the growth of pathogenic bacteria [26]. Nitrate-reducing starter cultures such as *Staphylococcus carnosus*, *Lactobacillus plantarum*, and *Lactobacillus sakei* are widely used to convert nitrate to nitrite [27]. According to the International Scientific Association of Probiotics and Prebiotics, prebiotics are considered dietary ingredients that are selectively utilized by beneficial microorganisms and provide health benefits [28]. Most prebiotics are carbohydrates with various molecular structures that are naturally present in the human diet. Prebiotics stimulate the growth of beneficial intestinal bacteria such as *Bifidobacterium* and *Lactobacillus* [29]. Common types of

prebiotics include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and lactulose [30]. Inulin improves various diseases by regulating carbohydrate, lipid, and amino acid metabolism, as well as intestinal, immune, and systemic immunomodulatory effects [31]. Therefore, strategies to inhibit dietary NA intake have been developed and prebiotics, probiotics, and phytochemicals have been used. The aim of the present study was to determine NA formation in cooked meat products by *in vitro* digestion and to inhibit NA concentrations and formation by adding blueberry, curcumin, prebiotic, and probiotic to fried meat products.

2. Materials and Methods

2.1. Materials

Alpha-amylase (1.5 U/mg, from *Aspergillus oryzae* powder), lipase (100–500 U/mg protein, from porcine pancreas Type II), pancreatin (from porcine pancreas 8 × USP specifications), pepsin (≥250 U/mg solid, from porcine gastric mucosa, lyophilized powder), NaCl, CaCl₂·2H₂O, urea, uric acid, bovine serum albumin, KCl, mucin, NaHCO₃, and bile salts mixture were used in the present study. These materials were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade chemicals were utilized.

NDMA (5000 µg/mL in methanol), NDEA (5000 µg/mL in methanol), N-nitrosomorpholine (NMOR, analytical standard), N-nitrosomethylethylamine (NMEA, analytical standard), N-nitrosopyrrolidine (NPYR, analytical standard), N-nitrosopiperidine (NPIP, analytical standard), sodium hydroxide (NaOH, ≥98%), and sodium acetate (CH₃COONa × 3H₂O, ≥99.0%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, gradient grade, ≥99.9%), dichloromethane (DCM, ≥99.8%), hydrobromic acid (HBr, ≥ 48%), and sodium bicarbonate (NaHCO₃, ≥99.7%) were obtained from Honeywell (Charlotte, NC, USA). Glacial acetic acid (CH₃COOH, 99.9%) was purchased from AppliChem (Darmstadt, Germany), dansyl chloride was obtained from Sigma-Aldrich (BioReagent, ≥99%), and boric acid (H₃BO₃, reagent grade) and acetone (analytic grade) were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Labconco System by Millipore (Bedford, MA, USA).

To eliminate any residual NAs or secondary amines in all glassware used in the experiment, it was carefully washed in two steps with sonication in deionized water at 80°C for 30 min and sonication in DCM for 15 min before analysis.

2.2. Preparation of Samples

Chicken legs, turkey neck, beef breast, and salmon were used. In March 2024, beef, turkey, and chicken meat were supplied from butchers and a salmon from fishermen. Supplements containing inulin and fructooligosaccharide as prebiotic; supplements containing various strains of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* with a total of 10×10⁹ colony forming unit (CFU) bacteria as probiotic (Table 1); and curcumin extract and blueberry

extract from phytochemicals were included in the study. Supplementary foods were supplied by pharmacies and online sales platforms.

Table 1. Bacterial Content and Amounts of Probiotic Supplement

Bacterial Content	Quantities
<i>Lactobacillus rhamnosus</i>	1×10 ⁹ CFU*
<i>Lactobacillus acidophilus</i>	1×10 ⁹ CFU*
<i>Lactobacillus gasseri</i>	1×10 ⁹ CFU*
<i>Lactobacillus reuteri</i>	1×10 ⁹ CFU*
<i>Lactobacillus plantarum</i>	1×10 ⁹ CFU*
<i>Lactobacillus fermentum</i>	1×10 ⁹ CFU*
<i>Bifidobacterium bifidum</i>	1×10 ⁹ CFU*
<i>Bifidobacterium longum</i>	1×10 ⁹ CFU*
<i>Bifidobacterium infantis</i>	1×10 ⁹ CFU*
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	750×10 ⁶ CFU*
<i>Streptococcus thermophilus</i>	250×10 ⁶ CFU*

The chicken thigh, beef breast, and salmon were cooked in a non-preheated oven at 200°C until their internal temperatures reached 84, 75, and 80°C, respectively (35 min). The turkey neck was cooked in a Fissler pressure cooker at level 2 for 7 min and then in a 200°C oven for 40 min; its internal temperature was 87°C.

2.3. In Vitro Gastrointestinal Digestive System Method

NA formation in the meat products was determined *in vitro* by simulating the gastrointestinal tract. The *in vitro* digestion procedure was a modified version of the procedure described by Lee et al. [32]. The mouth, stomach, small intestine, and large intestine juice used in the simulated gastrointestinal system were prepared using enzymes and organic and inorganic chemicals as shown in Figure 1. The organic and inorganic components of each digestive enzyme were prepared in 500 mL of distilled water. Then each enzyme was mixed with the prepared solution. The pH of each solution was adjusted to the appropriate value.

First, 5 g of samples were added to 100-mL Erlenmeyer flasks; then mouth, gastric, small intestine, and large intestine solutions were added to them, separately, and *in vitro* digestion was performed. In the oral phase, 5 mL of the prepared oral solution was added to the samples in a 100-mL beaker and homogenized. Then this mixture was incubated in a shaking water bath at 37°C for 5 min.

In the gastric phase, 12 mL of gastric solution was added to the mixture coming from the mouth phase and incubated for 2 h at 37°C in a shaking water bath.

In the small intestine phase, 10 mL of small intestine solution and 5 mL of bile solution were added to the mixture obtained after the gastric phase. This mixture was incubated again in a shaking water bath at 37°C for 2 h.

In the large intestine phase, 10 mL of the large intestine solution was added to the indigestible part after the small intestine phase and incubated at 37°C for 4 h. After the digestion process ended, the final volume was completed to 50 mL with deionized water. Then the samples were centrifuged at 8000 rpm for 10 min and filtered through a 0.45-µm filter and injected into high performance liquid chromatography (HPLC) system.

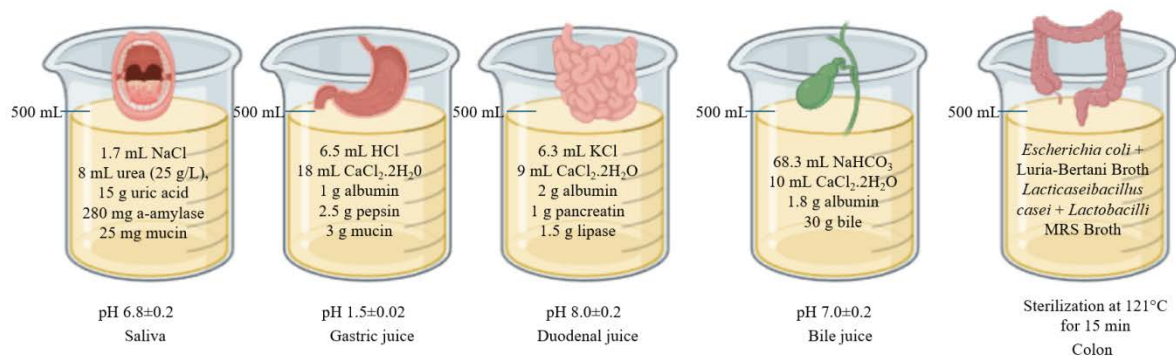


Figure 1. In vitro Simulated Human Gastrointestinal System.

2.4. Nitrosamine Analysis

The analysis of N-nitroso compounds was performed as follows. This is a modified version of the method described by Komarova & Velikanov [33].

2.5. Preparation of Solutions

Nitration solution (denitrosation solution) was prepared by dissolving 1 mL of HBr acid in 10 mL of acetic acid.

Dansyl chloride solution was prepared by dissolving 10 mg of dansyl chloride in 20 mL of acetone.

Sodium bicarbonate buffer solution was prepared by dissolving 0.32 g of NaOH and 2 g of NaHCO_3 in pure water (pH 10.5) and the volume was completed to 100 mL.

2.6. Preparation of Sample

After adding 25 mL of DCM to 5 g of sample, it was vortexed and centrifuged at 8000 rpm for 5 min. Then 0.1 mL of the supernatant was placed in a glass tube and 15 μL of nitration solution was added. It was kept in the dark at room temperature for 30 min. It was evaporated under nitrogen gas at 40°C and 400 μL of sodium bicarbonate buffer and 200 μL of dansyl chloride solution were added. After being kept at 40°C for 30 min, 400 μL of pure water was added. It was filtered through a 0.45- μm filter and injected into the HPLC system. The NDMA and NDEA forms of NA species detected in the study were well separated using the HPLC method with retention times of 5.5 and 7.5 min, respectively. The HPLC chromatogram of turkey of the meat products is shown in Figure 2.

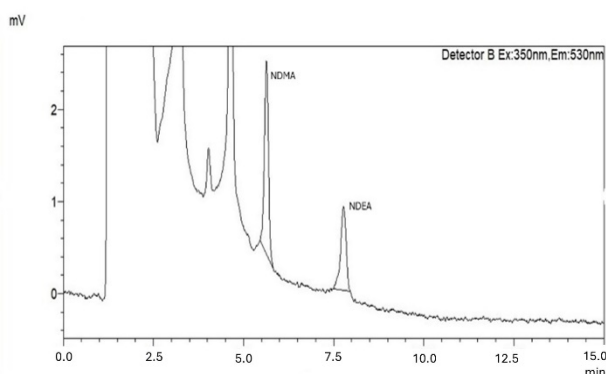


Figure 2. Nitrosamine Sample Chromatogram

2.7. Preparation of Standards

The standard solutions of NAs, NDMA, NDEA, NMOR, NMEA, NPYR, and NPIP were dissolved in DCM to prepare the stock standard. Then 0.1 mL of the stock standard was placed in a test tube and 25 mL of DCM was added to dissolve it. Next, 0.1 mL was put in the test tube and 15 μL of nitration solution was added and it was kept in the dark at room temperature for 30 min. After evaporation under nitrogen gas at 40°C, 400 μL of sodium bicarbonate buffer and 200 μL of dansyl chloride solution were added. After being kept at 40°C for 30 min, 400 μL of pure water was added. It was filtered through a 0.45- μm filter and injected into the HPLC system. The NA standard chromatogram is shown in Figure 3.

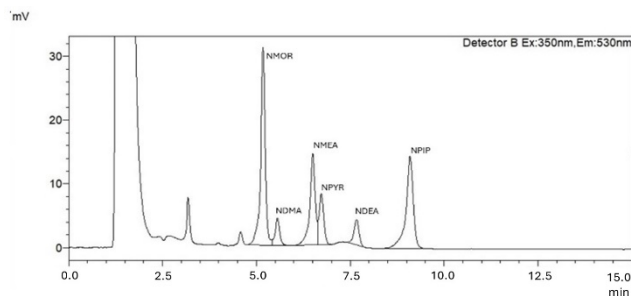


Figure 3. Nitrosamine Standard Chromatogram

2.8. HPLC Conditions

The HPLC analysis was performed using a Shimadzu Nexera-i HPLC system, and a Shimadzu RF-20A fluorescence detector (Shimadzu Corporation, Kyoto, Japan) was used for the separation of NDMA, NDEA, NMOR, NMEA, NPYR, and NPIP. The mobile phase consisted of 66.6% acetonitrile and 33.3% deionized water. The separation was done with an Eclipse X08-C18, 5 μm , 4.6×150 mm column (Agilent, USA) with a flow rate of 1.4 mL/min and injection volume of 10 μL . The fluorescence detector excitation and emission wavelengths were 350 and 530 nm, respectively. The column oven temperature was 40°C.

2.9. Statistical Analysis

All analyses conducted were performed in triplicate. Significant differences between the results were shown

by analysis of variance (ANOVA) carried out by Minitab 17 ($p < 0.05$, Tukey's test).

3. Results and Discussion

In the present study, the effects of prebiotic, probiotic, and blueberry and curcumin extracts were investigated in different meat products. The names and abbreviations of these samples are given in Table 2.

Our study contributes to the literature by determining the NA amounts of unprocessed cooked meat products and examining their formation after cooking and digestion. While it is stated in the literature that unprocessed and uncooked meats contain trace amounts of NAs, it is emphasized that NAs increase after cooking these foods (baking, frying, grilling, and microwave cooking). This shows that cooking produces NAs [34]. In addition, NA data on cooked unprocessed meat and fish are limited [5].

Table 2. N-Nitrosamine values of samples before and after *in vitro* digestion.

Code of samples	Name of samples	Predigestion			Post-digestion			% Decrease Rate
		NDMA ($\mu\text{g/kg}$)	NDEA ($\mu\text{g/kg}$)	NDMA+NDEA ($\mu\text{g/kg}$)	NDMA ($\mu\text{g/kg}$)	NDEA ($\mu\text{g/kg}$)	NDMA+NDEA ($\mu\text{g/kg}$)	
B	Beef	0.253 $\pm$ 0.010 ^a	0.033 $\pm$ 0.001 ^a	0.286 $\pm$ 0.010 ^a	0.438 $\pm$ 0.018 ^b	0.090 $\pm$ 0.004 ^b	0.528 $\pm$ 0.019 ^b	85
C	Chicken	0.214 $\pm$ 0.006 ^a	0.053 $\pm$ 0.002 ^a	0.267 $\pm$ 0.009 ^a	0.396 $\pm$ 0.012 ^b	0.139 $\pm$ 0.004 ^b	0.535 $\pm$ 0.019 ^b	100
T	Turkey	0.153 $\pm$ 0.009 ^a	0.095 $\pm$ 0.006 ^a	0.248 $\pm$ 0.009 ^a	0.563 $\pm$ 0.034 ^b	0.138 $\pm$ 0.008 ^b	0.701 $\pm$ 0.025 ^b	183
S	Salmon	0.109 $\pm$ 0.005 ^a	0.050 $\pm$ 0.003 ^a	0.159 $\pm$ 0.006 ^a	0.583 $\pm$ 0.029 ^b	0.079 $\pm$ 0.004 ^b	0.662 $\pm$ 0.023 ^b	316
BE	Blueberry Extract	ND	ND	ND	ND	ND	ND	
CE	Curcumin Extract	ND	ND	ND	ND	ND	ND	
I	Inulin	ND	ND	ND	ND	ND	ND	
P	Probiotic	ND	ND	ND	ND	ND	ND	

Values are means $\pm$ standard deviation ($n = 3$). Different letters within the same group in each row indicate significant differences between treatments (ANOVA $p < 0.05$, Tukey's test). NDMA: N-nitrosodimethylamine; NDEA: N-nitrosodiethylamine

The NA values of the unprocessed cooked meat products are given in Table 2. In predigestion cooked meat products, the amount of NDMA was 0.109–0.253 $\mu\text{g/kg}$ and the amount of NDEA was 0.033–0.095 $\mu\text{g/kg}$. It has been reported that NAs can also be found in unprocessed and nitrite-free meats [35], and this view is supported by the present study. The amounts of NAs in unprocessed cooked meat products are given in Table 2. In predigestion cooked meat products, the amounts of NA determined were NDMA 0.109–0.253 $\mu\text{g/kg}$ and NDEA 0.033–0.095 $\mu\text{g/kg}$. Rywotycski [35] supports our study by indicating that NAs can also be found in unprocessed and nitrite-free meats.

The Panel on Food Additives and Nutrient Sources reviewed studies to determine the exposure of consumers to the most common volatile NAs (NDMA and NDEA) in meat products found in their diets. The Panel reported that the total amount of NAs was less than 5.5 $\mu\text{g/kg}$. The average amount of NDMA was 0.7–2.7 $\mu\text{g/kg}$ and of NDEA was 0.04–0.9 $\mu\text{g/kg}$ [8]. These findings are similar to the results of the current study.

In the present study, NA formation in beef was higher than that in chicken and turkey. Differences in the amount of NAs contained in meat products may be due to the nutrients and their ratios found in meat. A study on sausage types showed that the amounts of NAs in meat sausage samples were higher than those in chicken sausage samples [36]. The researchers suggested that this was due to the role of various components of meat in the formation of NAs.

In the present study, the amount of NA formation in chicken was 0.267 $\mu\text{g/kg}$ and it contained higher NAs than turkey and salmon and lower NAs than beef. Poultry is a major source of dietary exposure to volatile NAs for the US population (~21%). Higher levels of volatile NAs

(NDMA, NPYR, and NPIP) were detected in spicy and grilled poultry products. Spice use can lead to the formation of NPYR and NPIP in these products [37].

In our study, 0.253 $\mu\text{g/kg}$ NDMA was detected in the cooked beef. Unlike the current study, NDMA (5–15 $\mu\text{g/kg}$) and NDEA (5–20 $\mu\text{g/kg}$) were detected in studies conducted on beef and other red meats [38]. Factors such as regional differences, production methods, and cooking habits explain the difference in NA amounts of similar meat products from the findings in other studies [39].

In the present study, NAs were detected in salmon after cooking; the amount of NDMA was 0.109 $\mu\text{g/kg}$ and the amount of NDEA was 0.050 $\mu\text{g/kg}$. In the literature, the potential for NDMA formation in fish is explained by the high levels of NDMA precursors such as dimethylamine and trimethylamine [40]. Studies have investigated the effects of different cooking processes on NA formation in fish and it has been stated that NAs increase with cooking processes in fish [6,40].

In the current study, NAs were detected in all meat products cooked at 200°C. The effects of cooking temperature on NA formation in meat products were investigated. It has been stated that higher temperatures cause more nitrite to convert into NAs [41,42]. In this context, recent studies do not recommend overcooking to reduce nitrite in meat and meat products [18].

In our study, NA formation was detected in all meat products that were roasted in the oven at 200°C. It is thought that NA formation increases as a result of high-temperature cooking. Unlike other meats, turkey neck was first boiled for 10 min and then roasted in the oven at 200°C. It was determined that turkey formed less NDMA and NDEA compared to beef and chicken. Among the cooking methods, boiling can reduce the formation of NAs because they can dissolve in the cooking water. In

addition, keeping the cooking time short provides inhibition of NA formation. The boiling points of NAs are between 150°C and 220°C and the formation of NAs can be prevented by the evaporation of these volatile compounds [11]. It is thought that the lower NA content of turkey in the current study may have been due to the boiling process applied differently from the other meats.

In our study, a significant increase in NA amounts was found in meat products after *in vitro* digestion. An increase of 85% was observed in beef, 100% in chicken, 183% in turkey, and 316% in salmon (Table 2). NAs can also be produced endogenously by consuming meat products [31]. Endogenous N-nitrosation reactions between NA precursors and nitrogenous residues in the gastrointestinal tract provide the strongest source of NA exposure in humans [13]. Proteolysis during digestion provides essential amino acids, especially together with nutrients, to the nutritional quality of meat and meat products. The produced peptides may also play a role in the formation of nitroso compounds by reacting nitrite with secondary amines to form NAs [34]. Due to this effect of digestion, the amounts after digestion should be taken into account to assess NA exposure from meat products.

In order to evaluate the relationship between NA formation in meat products and the amount of iron in meat, the iron amounts obtained from the USDA database of meats were compared with the NA amounts in the current study and a significant relationship was found [43]. As the amount of iron in meat increased, an increase in the amounts of NAs formed was also observed (Table 3). Moreover, free iron, which is abundant in the intestine, is the main catalyst for nitrosation [44]. There is a significant and consistent relationship with previous studies showing the relationship between heme iron intake from meat products and NA formation [45]. In this context, the increase in NA amounts in cooked meat products after digestion is based on the gastrointestinal tract conditions and especially the changing pH [46].

Table 3. Iron Amounts in Meat Products

Meat Products	Iron (Fe) mg/100 g
Beef	1.64
Chicken Thighs	0.94
Turkey	0.8
Salmon	0.26

The amounts of NDMA and NDEA in the meat samples after *in vitro* digestion are given in Table 4. While NDMA was 0.438 µg/kg and NDEA was 0.090 µg/kg in beef after *in vitro* digestion, after *in vitro* digestion by adding supplements NDMA decreased to 0.233–0.415 µg/kg and NDEA decreased to 0.015–0.083 µg/kg. Blueberry extract provided the greatest reduction in beef. While NDMA was 0.396 µg/kg and NDEA was 0.139 µg/kg in chicken after *in vitro* digestion, after *in vitro* digestion by adding supplements NDMA decreased to 0.167–0.290 µg/kg and NDEA decreased to 0.018–0.072 µg/kg. In chicken, the greatest reduction was provided by inulin and probiotic. In turkey, after *in vitro* digestion, NDMA was 0.563 µg/kg and NDEA was 0.138 µg/kg; after *in vitro* digestion by adding supplements, NDMA decreased to 0.160–0.396 µg/kg and NDEA decreased to 0.015–0.075 µg/kg. In salmon, the greatest reduction was provided by blueberry extract in NDMA and by inulin in NDEA.

µg/kg and NDEA decreased to 0.030–0.090 µg/kg. In turkey, the greatest reduction was provided by blueberry extract in NDMA and by inulin in NDEA. In salmon, after *in vitro* digestion, NDMA was 0.583 µg/kg and NDEA was 0.079 µg/kg; after *in vitro* digestion by adding supplements NDMA decreased to 0.185–0.417 µg/kg and NDEA decreased to 0.015–0.075 µg/kg. In salmon, the greatest reduction was provided by blueberry extract in NDMA and by inulin in NDEA.

Table 4. Effects of supplements on Nitrosamine formation in the *in vitro* gastrointestinal digestive system.

Samples	Postdigestion	
	NDMA (µg/kg)	NDEA (µg/kg)
B	0.438±0.018 ^a	0.090±0.004 ^a
B+BE	0.233±0.009 ^c	0.015±0.001 ^e
B+CE	0.415±0.017 ^a	0.031±0.001 ^d
B+I	0.354±0.014 ^b	0.083±0.003 ^b
B+P	0.335±0.013 ^b	0.072±0.003 ^c
C	0.396±0.012 ^a	0.139±0.004 ^a
C+BE	0.290±0.009 ^b	0.031±0.001 ^d
C+CE	0.200±0.006 ^c	0.072±0.002 ^b
C+I	0.167±0.005 ^d	0.018±0.001 ^e
C+P	0.167±0.005 ^d	0.044±0.001 ^c
T	0.563±0.034 ^a	0.138±0.008 ^a
T+BE	0.160±0.010 ^d	0.083±0.005 ^{bc}
T+CE	0.396±0.024 ^b	0.090±0.005 ^b
T+I	0.329±0.020 ^c	0.030±0.002 ^d
T+P	0.313±0.019 ^c	0.075±0.005 ^c
S	0.583±0.029 ^a	0.079±0.004 ^a
S+BE	0.185±0.009 ^d	0.016±0.001 ^b
S+CE	0.417±0.021 ^b	0.075±0.004 ^a
S+I	0.217±0.011 ^d	0.015±0.001 ^b
S+P	0.283±0.014 ^c	0.017±0.001 ^b

Values are means ± standard deviation (n = 3). Different letters in each meat group indicate significant differences between treatments (ANOVA p < 0.05, Tukey's test). B: Beef; C: Chicken Thighs; T: Turkey; S: Salmon; +BE: Treated with blueberry extract; +CE: Treated with curcumin extract; +I: Treated with prebiotic supplement; P: Treated with probiotic supplement; NDMA: N-nitrosodimethylamine; NDEA: N-nitrosodiethylamine.

Figure 4a shows the amounts by which supplements reduce NDMA formation in meat products in the *in vitro* gastrointestinal digestive system. The greatest reductions in beef, turkey, and salmon were provided by blueberry extract and by inulin in chicken.

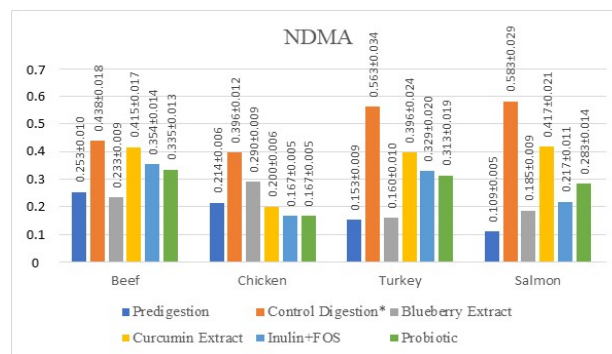


Figure 4a. Amounts of supplements that reduce NDMA formation in the *in vitro* gastrointestinal tract (NDMA: N-nitrosodimethylamine; NDEA: N-nitrosodiethylamine) (*Control digestion: *in vitro* digestion without added supplements).

Figure 4b shows the amounts by which supplements reduce NDEA formation in meat products in the *in vitro* gastrointestinal digestive system. The greatest reduction in beef was provided by blueberry extract and by inulin in chicken, turkey, and salmon.

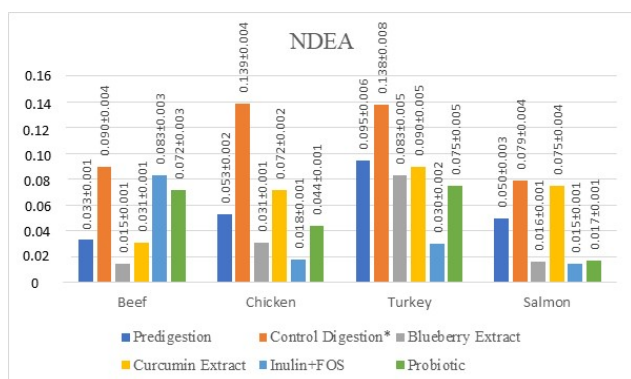


Figure 4b. Amounts of supplements that reduce NDEA formation in the *in vitro* gastrointestinal digestive system (NDMA: N-nitrosodimethylamine; NDEA: N-nitrosodiethylamine) (*Control digestion: *in vitro* digestion without added supplements).

3.1. Inhibition of N-Nitroso Compounds by Blueberry

In the present study, herbal products were used to inhibit NA formation. The strategy that distinguishes our study from others is the inhibition of NAs formed endogenously by the digestion of meat products by applying blueberry, curcumin, prebiotic, and probiotic. Meat products decarboxylate amino acids to produce biogenic amines. Currently, there are several strategies to reduce NA and biogenic amine production in foods, including reduction of NA precursors, inhibition of reactions leading to NA formation, and direct degradation approaches [47]. Processing or consumption of plant products with meat products is among these strategies [48]. The use of ingredients containing high levels of polyphenols [49] in meat formulations inhibited N-nitroso compounds. In a study in which various herbal products were added to bacon, the addition of green tea and grape seed extract (proanthocyanidins and catechins) reduced the amount of NDMA found in dry-cured bacon [50]. It is recommended to add substances rich in phenolic acids, flavonoids, tannins, stilbenes, punicalagins, and ellagic acid to foods [51].

In our study, blueberry extract added to meat products inhibited NA formation by 27%–72% in the *in vitro* digestive system. Blueberry extract showed stronger effects compared to prebiotic, probiotic, and curcumin extract in the meat products (except chicken). Adding antioxidants to meat products is a common way to reduce harmful metabolites. Commonly used antioxidants include polyphenols and vitamins [39]. Studies indicate that adding phenolic compounds to meat products increases their antioxidant activity during digestion [19]. In this sense, blueberry can control the formation of NA compounds that are harmful to health and have carcinogenic potential in consumed meat products. In a study examining the effect of 15 types of blueberries on nitrite and NAs in cooked ham products, the inhibition rate of blueberry in NA formation was 63% on average.

This shows that blueberry has a significant inhibitory effect on nitrite and NAs in cooked ham products [14].

In the present study, blueberry and curcumin extracts used as antioxidants showed an inhibitory effect on NA formation. Moreover, ascorbate (pH 5.0–6.0) used in processed meat products as an antioxidant reduces Fe^{+3} in metmyoglobin to Fe^{+2} in deoxymyoglobin [52]. The inhibitory effect of antioxidants can be explained by the iron mechanism mentioned above.

3.2. Inhibition of N-Nitroso Compounds by Prebiotic

In our study, the addition of inulin to meat products reduced postdigestive NA formation. Inulin was one of the most effective supplements that could inhibit postdigestive NA formation in chicken, turkey, and salmon *in vitro*.

Prebiotics beneficially affect the host by selectively stimulating the growth and activity of one or several bacterial species in the colon [53]. They act as “nutrients” for beneficial bacteria in the gut, helping to increase their numbers and support a healthier microbiome [54]. Inulin-type prebiotics as dietary fiber interventions have attracted attention due to their proven nutritional, therapeutic, and preventive properties [55]. Additionally, studies have shown the use of inulin in the development of therapeutic/functional meat products (processed meat products such as beef burgers, fermented sausages, and frankfurters) [56,57,58].

Studies have shown that fortification of pork sausage with inulin may be a strategy to inhibit endogenously formed NAs after consumption [59]. The inclusion of inulin in meat products improves the functional and health properties of meats [60]. However, comprehensive studies on the use of inulin as a prebiotic in meat products are quite limited.

In the present study, the addition of prebiotic to meat products reduced NA formation in the colon *in vitro* digestive system. In a study conducted on experimental animals, red meat increased bacterial nitrate-reductase activity. Dietary fiber components such as wheat bran, cellulose, and pectin suppress this activity [61]. The potential inhibitory effect of the prebiotic supplement containing inulin+FOS used in the current study on NA formation is explained by this situation.

3.3. Inhibition of N-Nitroso Compounds by Probiotic

Among the modifiable factors affecting the composition and functions of the intestinal microbiota, diet plays an important role [62]. In the present study, the effect of dietary supplements on the formation of endogenously occurring NAs in different meat products was investigated in the *in vitro* gastrointestinal system. Probiotics may support intestinal health by regulating microbiota, stimulating the immune system, improving nutrient bioavailability, and reducing the risk of various diseases [63]. During hydrolysis, several of these probiotics produce enzymes that increase protein and fat absorption. For example, LAB has been shown to increase the concentration of vitamin B complex in fermented foods

[64]. Moreover, probiotics have significant effects on the production of enterocytes by neutralizing the activity of dietary carcinogens (NAs). Therefore, the addition of probiotic bacteria to meat products preserves the functional properties of meat by inhibiting the formed NA potential [65]. Additionally, it is indicated that LAB may inhibit NA accumulation through direct adsorption or metabolism [52].

In our study, attention was paid to the selection of probiotics so that the colony quantity and diversity of *Lactobacillus* strains were high (1×10^9) (Table 1). Little is known about the interaction of probiotic bacteria with NDMA. It was concluded that NDMA concentrations (2–100 µg/mL) did not affect the growth or viability of *Lactobacillus* strains and they remained viable and active even in the presence of high NDMA concentrations [66].

In the present study, the addition of probiotic supplements containing various strains to different meat products in the *in vitro* digestive system inhibited NA formation by 20%–79% (Table 5). This difference in inhibition rates was affected by the content of the meat products and the bacterial strains. The highest inhibition occurred in NDEA in salmon; the lowest inhibition occurred in NDEA in beef. The literature emphasizes that for bacterial NA inhibition there must be bacterial strains with nitrate and nitrite reducing activities. In a study examining NA formation in the large intestine, it was shown that NA formation depends on the presence of intestinal flora and is affected by dietary nitrate [67].

The probiotic supplement used in the present study inhibited NDMA levels in meat products by 23%–58% and contained 1×10^9 CFU *Lactobacillus plantarum*. Similar to the present study, it was found that sausages with *L. plantarum* added inhibited the formation of bioamines, which are precursors of NAs, and significantly inhibited the amounts of NAs in sausages [68]. In another study, the effect of fermentation by four strains (*L. carnosum*, *L. mesenteroides*, *L. plantarum*, and *L. sakei*) in the presence of meat products in the gastrointestinal tract on the formation of NDMA and its precursors was investigated [69]. Among the bacteria, the greatest effect was suggested to be exerted by *L. plantarum* and *L. sakei*. In addition, it was reported that these strains could reduce the NDMA content by up to 50% by inhibiting NDMA synthesis [69]. That study is consistent with the current study in terms of its capacity to inhibit NDMA.

In the current study, probiotic supplementation showed the greatest effect by inhibiting NA formation by 79% in salmon (Table 5). In this context, the effect of fish products and probiotics is thought to be due to the potential effect of *Lactobacillus plantarum*. In other studies, the addition of *L. plantarum* to fish inhibited the accumulation of amines and their precursors during fish processing [70,71]. Similarly, the effect of adding *L. plantarum* 120 on NDMA formation during *in vitro* digestion in fermented fish products was investigated. Inhibition was observed after the addition of *L. plantarum* 120 to fermented fish, which had an NDMA amount of 5.39 µg/kg after digestion and was determined to be 2.12 µg/kg [48]. These results are consistent with the inhibition rate of *L. plantarum* in the current study.

The present study was conducted to better understand the potential effect of probiotics in inhibiting the

formation of carcinogenic NAs in meat products. Probiotic supplementation containing *L. gasseri* inhibited NDMA levels in meat products by 23%–58% (Table 5). Similarly, the use of probiotics (*Lactobacillus gasseri* OLL2716: LG21) was investigated to regulate the intestinal flora of colorectal cancer patients and prevent colorectal carcinogenesis. It has been concluded that when probiotics are taken by colorectal cancer patients, the intestinal environment improves and colorectal carcinoma can be prevented with probiotics [72].

Further research is needed to investigate new LAB strains for their ability to inhibit NA levels and to examine their potential interactions with meat products in the gastrointestinal tract. The present study contributes to this.

Table 5. Inhibition rates of Nitrosamine formation in the gastrointestinal digestive tract *in vitro* by supplements

Eg.	BE		CE		I		P	
	NDMA	NDEA	NDMA	NDEA	NDMA	NDEA	NDMA	NDEA
B	47±4 ^a	83±7 ^a	5±0 ^c	65±6 ^b	19±2 ^b	8±1 ^d	23±2 ^b	20±2 ^c
C	27±2 ^b	78±7 ^{ab}	49±4 ^a	48±4 ^c	58±5 ^a	87±7 ^a	58±5 ^a	69±6 ^b
T	72±6 ^a	40±3 ^b	30±3 ^c	34±3 ^b	42±4 ^b	78±7 ^a	44±4 ^b	45±4 ^b
S	68±6 ^a	80±7 ^a	29±2 ^c	5±0 ^b	63±5 ^{ab}	81±7 ^a	51±4 ^b	79±7 ^a

Values are means ± standard deviation (n = 3). Different letters within the same group in each row indicate significant differences between treatments (ANOVA $p < 0.05$, Tukey's test). B: Beef; C: Chicken Thighs; T: Turkey; S: Salmon; BE: Blueberry Extract; CE: Curcumin Extract; I: Prebiotic supplement; P: Probiotic supplement; NDMA: N-nitrosodimethylamine; NDEA: N-nitrosodiethylamine

3.4. Inhibition of N-Nitroso Compounds by Curcumin

In the present study NA formation was inhibited by 5%–65% in meat products to which curcumin extract was added. Among the meat products, curcumin showed the highest inhibition of NDMA and NDEA in chicken and the least effect in salmon (Table 5). Curcumin, the active ingredient of turmeric, is a powerful antioxidant. Various studies on animal models have shown that curcumin suppresses carcinogenesis in different organs [38]. In addition, curcumin significantly reduces serum levels of IL-2, IL-6, ALT, and malondialdehyde [48].

Curcumin inhibited the effects of blueberry, prebiotic, and probiotic to a lesser extent in the current study. This is explained by the low bioavailability and limited pharmacological potential of curcumin after oral intake [73].

In the current study, the NA inhibitory effect of curcumin extract was more limited compared to blueberry extract, which is explained by the piperidine found in curcumin extract. In addition, secondary amines such as piperidine can be nitrosated, albeit slowly, without requiring heat treatment. Therefore, the use of spices and herbs containing alkaloids and nitrates can be considered as an additional source of NAs in meat products [11].

4. Conclusion

NAs can be considered as a criterion for the evaluation of the nutritional quality of meat products subjected to different heat treatments. The aim of our study was to determine the amounts of NAs formed by cooking

different meat products and to inhibit the amounts of NAs formed in these products. Blueberry, curcumin, prebiotic, and probiotic supplements were used for the inhibition of the amount of NA formed in the meat products. The interactions of blueberry, curcumin, prebiotic, and probiotic consumed together with meat during digestion and their effects on NA formation were reviewed. It was found that NA formation increased in the cooked meat products in the digestive system and the supplements used had the capacity to effectively inhibit this formation. However, further *in vitro* and *in vivo* studies are needed to fully explain the inhibition mechanism of bioactive compounds in supplements.

ACKNOWLEDGMENTS

The authors would like to thank all the participants. This study is part of H.S.S.M.'s doctoral thesis "Investigation of the Effects of Adding Prebiotic, Probiotic and Phytochemicals to Some Meat Products on the Formed Metabolites by Gastrointestinal Simulation"

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