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Research Article

The Effect of the Combination of Rosemary Extract and Green Tea Extract on Nitrosamine Content, Microbiological, Physicochemical and Sensorial Properties of Heat-Treated Sucuk

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ABSTRACT

In this study, it was aimed to determine the effect of using rosemary extract together with green tea extract (RE/GTE) on nitrosamine content and quality characteristics of heat-treated sucuk. In addition, the influence of cooking time on the formation of nitrosamines was also investigated. Four different batters were prepared as follows: control (C), 0.1% RE/GTE (RG1), 0.2% RE/GTE (RG2), and 0.3% RE/GTE (RG3). The use of RE/GTE caused a decrease in lactic acid bacteria and *Micrococcus/Staphylococcus* counts in the final product. While the use of RE/GTE did not affect the a_w value, the lowest pH value (4.80±0.05) was observed in the RG3 treatment. RE/GTE resulted in a reduction in residual nitrite, and the lowest level was determined as 12.60±0.87 mg/kg. The lower mean TBARS values were determined in the RG2 and RG3 treatments. RE/GTE did not affect the L* and a* values, however, b* value increased in the RG2 and RG3 treatments. The lowest odor, taste, and general acceptability scores were determined in the RG3 group. The use of RE/GTE had no significant effect on nitrosodiethylamine, nitrosodimethylamine, nitrosopiperidine and nitrosopyrrolidine. The levels of nitrosamines increased with cooking, but the interaction of RE/GTE and cooking time was not significant. RE/GTE treatments also caused a decrease in hexanal level and an increase in some terpene compounds.

Keywords: Fermented sausage, Green tea, Heat treated sucuk, Nitrosamine, NDMA, NDEA, NPIP, Rosemary

Introduction

Fermented sausages are widely produced meat products around the world which have different properties. Characteristic properties of these products are affected by such factors as the species and breed of animal, type of fat, degree of comminution, additives (sugar, salt, spices, curing agent), starter culture, type of casing used, fermentation and ripening/drying conditions [1]. Sucuk is a dry-fermented type of sausage that is popular in Türkiye, which is produced by using beef, water buffalo meat, and, although rarely, mutton. In addition to meat fat, sheep tail fat is also used as a source of fat. Typical spices are allspice, paprika, cumin, black pepper and, especially, garlic. The main steps in sucuk production process are preparation of sucuk batter, fermentation and ripening/drying [2,3]. Another type of fermented sausage produced in Türkiye is heat-treated sucuk (HTS). The formulation of this product is same as sucuk. However, after a short fermentation time (initial temperature 22-25°C, pH value <5.3 during fermentation), heat treatment is applied, which is followed by the drying phase [4,5]. The moisture : protein ratio and fat : protein ratio for this product must be under 3.6:1 and 2.5:1, respectively, whereas pH of the product must be at most 5.6 [6].

HTS is an industrial product and only nitrite (150 mg/kg at max) is allowed in the production [7]. Besides its antioxidant and antimicrobial properties, this curing agent also plays a positive role in formation of color and aroma, yet it is also an important factor in the formation of nitrosamines with known carcinogenic effects [8]. The level of nitrosamines in HTS and other fermented sausages

may be below the detectable limit, or may reach high levels [8-14]. The lipid and protein degradation products formed during ripening stage of fermented sausages may be a good source of nitrosamine formation [15]. As a matter of fact, Sallan [16] reported that sheep tail fat increases oxidation and is effective on nitrosamines because it contains high unsaturated fat. Many factors such as the presence of inhibitors and catalysts, cooking temperature and time, cooking method, presence of microorganisms with decarboxylase activity, residual nitrite level, and presence of precursor substances are also effective in the formation of nitrosamines [8,13,17]. In a study conducted on HTS, the effects of nitrite levels, cooking degree, the use of ascorbic acid and starter cultures on nitrosamine formation were examined and it was concluded that cooking time was the most important factor among the factors examined in terms of nitrosamines [10]. Unlike many types of fermented sausage, HTS is cooked before consumption, thus strategies to prevent nitrosamine formation in this product are of great importance.

Rosemary *(Rosmarinus officinalis* L.) and green tea *(Camellia sinensis* L.) extracts are products used in meat products for their antioxidant activities [18-21]. There is little information about the effect of these natural products on the formation of nitrosamines in meat products [22-24]. Li et al.[22] investigated the effects of green tea and grape seed polypheneols on nitrosamine formation in dry cured sausage. In the study on dry cured bacon, the effects of green tea polyphenols, grape seed extract and their combination on N-nitrosodimethylamine (NDMA) were determined [23]. Another study was carried out in cooked sausage (western-style smoked sausage) [24]. In this study, the effects of rosemary and green tea extract, which are used as antioxidants in meat products and have an important place among natural antioxidants, on nitrosamine formation, volatile compounds, microbiological, physicochemical and sensory properties in HTS were investigated.

Material and Methods

Material

For the production, lean meat and beef fat were used as raw material. The supply of raw materials was carried out at three different times, and three productions were made. The *Latilactobacillus sakei* S15 and *Staphlococcus xylosus* GM92 [25,26] were used as starter cultures, and were added to the batters at 10^7 cfu/g and 10^6 cfu/g, respectively. Rosemary extract + green tea extract (RE/GTE) (Veg stable® 721) was obtained from a commercial company (Florida Food Products, USA).

Heat Treated Sucuk Production

In the manufacture of HTS, 80% beef meat and 20% fat were used. The formulation was included per kg meat and fat: 20 g NaCl, 2.5 g allspice, 9 g cumin, 5 g black pepper, 7 g red pepper, 4 g sucrose, and 0.15 g sodium nitrite. Four different batters of HTS were produced: control: HTS without rosemary extract + green tea extract (RE/GTE), RG1: 0.1% RE/GTE, RG2: 0.2% RE/GTE, and RG3: 0.3% RE/GTE. For each treatment, three batters were prepared at three different times.

The batters prepared using a laboratory-type cutter (Mado Typ MTK 662, Dornhan, Schwarzwald) were filled into collagen casings 38 mm in diameter, Naturin GmbH Co., Weinheim, Germany) by means of a filling machine (Mado Typ MTK 591, Dornhan, Schwarzwald). Then, samples were subjected to the fermentation in an automatic climate unit (Reich, Thermoprozestechnik GmbH, Schechingen, Germany) at 22±1°C and 90±2% relative humidity for 24 h. Following the stage, heat treatment was applied in a steam cooking chamber (Mauting, Valtice, Czech Republic) up to 64°C of core temperature. The samples were then dried in the automatic climate unit for 48 h at 16±1°C and 84±2% relative humidity.

Cooking Procedure

HTS sliced (thickness: 0.5 mm) was cooked on a hot plate preheated to 180°C. The cooking time was applied as 1 min (0.5 min per side) or 3 min (1.5 min per side). The uncooked samples were considered as the control group (0 min). The samples were homogenized and taken into glass jars and frozen at -20°C.

Microbiological Analyses

For the enumeration of lactic acid bacteria number, De Man Rogosa Sharpe Agar (MRS, Merck, Darmstadt, Germany) was used. The incubation was carried out at 30°C for 2 days in anaerobic jars (Anaerocoult A, Merck, Darmstadt, Germany). To determination of *Micrococcus/Staphylococcus* number, Mannitol Salt Phenol Red Agar (MSA, Merck) was used, and the incubation was carried out at 30°C for 2 days. For Enterobacteriaceae, Violet Red Bile Dextrose Agar (VRBD, Merck) plates were subjected to incubation at 30°C for 2 days in anaerobic jars (Anaerocoult A, Merck, Darmstadt, Germany) [4].

pH and a_w Analyses

For pH analysis, 10 g samples were homogenized with 100 mL of distilled water using ultra-turrax. The pH was measured with a pH meter (Mettler Toledo, Switzerland). A water activity device (TH-500 a_w Sprint, Novasina, Pfaffikon, Switzerland) calibrated at 25°C with 6 different salt solutions was used for the a_w analysis [27].

Residual Nitrite and TBARS Analyses

To determine residual nitrite, the method of NMKL[28] was used. For extraction, 10 g sample was mixed with 50 mL

ultrapure water (50-60°C) and then, 50 mL acetonitrile was added. After stirring for 15 min, the volume was made up to 200 mL with ultrapure water. After filtration, the samples were transferred to vials. The residual nitrite content was determined using high-performance liquid chromatography (HPLC)/diode array detector (DAD) (Agilent Technology, Santa Clara, CA, USA). The flow rate, UV wavelength and injection volume were used as 2 mL/min, 220 nm and 100 µL, respectively. Results were expressed in mg/kg based on the calibration curve prepared with nitrite standard.

The method given by Lemon [29] was applied in the analysis of thiobarbituric acid reactive substance (TBARS). 2 g of homogenized sample was mixed with 12 mL of TCA solution. After filtering the homogenate through a Whatman 1 filter, 3 mL of the filtrate was added to 0.02 M thiobarbituric acid solution. The mixture was kept in a boiling water bath for 40 min. The mixture was then centrifuged at 2000 G for 5 min and the absorbance was determined at 530 nm. The TBARS value was given as mg MDA/kg sample.

Color and SensoryAnalyses

The color values (L*, a* and b*) were determined using a chroma meter (Minolta, Osaka, Japan) [4]. The sensory analysis was carried out using a structured nine-point scale (1-9 scales: 1: "dislike extremely" to 9: "like extremely"). A total of 20 semi-trained panelists, consisting of 14 females and 6 males, participated in the sensory evaluation. Prior to analysis, panelists were briefed on the application of sensory analysis.

Volatile Compound Analyses

The vial containing 5 g of sample was placed in a thermal block (Supelco, USA) at 30°C for 1 h to collect the volatile compounds. In the extraction, solid phase microextraction with a carboxen/polydimethylsiloxane fiber (CAR/PDMS, 75 µm, Supelco, USA) was used, the fibre was placed in the vial and kept for 2 h. The gas chromatography/mass spectrometry (Agilent, Santa Clara, CA, USA) was used to identify of volatile compounds. The system conditions given by Kaban [30] were used and the libraries of the mass spectrometer and standard materials were used to evaluate the results. In addition, the standard mix (Parrafine mix, 44585-U, Bellefonte, PA, US) was used to determine the Kovats index. The results were given as AUx 10^6.

Nitrosamine Analyses

Nitrosamines were extracted according to the method specified by Wang et al.[23]. GC/MS (Agilent 6890 N/ Agilent 5973, USA) was used to detect nitrosamines. Helium was used as carrier gas and DB-5MS (30 m × 0.25 mm × 0.25 µm, Agilent, USA) as column, and the system was operated in SIM mode. The oven temperature program given by Sallan [16] was applied. Nitrosamine mix (EPA 521 nitrosamine Mix, Supelco, Bellefonte, PA, USA) was used for identification and nitrosamine levels (N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), N-Nitrosopiperidine (NPIP), N-Nitrosopyrrolidine(NPYR),Nitrosodipropylamine (NDPA), N-Nitrosomethylethylamine (NMEA) and N-Nitrosodibutylamine (NDBA)) were determined at µg/kg level. The limit of dedection (LOD) and the limit of quantification (LOQ) values were determined for NDMA (LOD = 0.32, LOQ = 0.97), NDEA (LOD = 0.37, LOQ = 1.12), NPYR (LOD = 0.37, LOQ = 1.13), and NPIP (LOD = 0.32, LOQ = 0.98).

Statistical Analyses

The experiments were carried out according to the randomized complete block design. The use of rosemary extract+green tea extract (RE/GTE) was evaluated as main effect, and the replicates as a random effect. For nitrosamines, RE/GTE and cooking time were also evaluated as main effects. The differences between the means were determined using Duncan's multiple range tests at the $P<0.05$ level. The statistical analyses were carried out using the SPSS version 20 statistical program (SPSS, Chicago, IL, USA). The principal component analysis (PCA) was also performed to determine the relationship between RE/GTE and volatile compounds, and between RE/GTE, cooking time and nitrosamine using Unscrambler program (CAMO version 10.1, Oslo, Norway).

Results

The effect of rosemary extract/green tea extract (RE/GTE) treatment on lactic acid bacteria (LAB), *Micrococcus/Staphylococcus*, pH, a_w, residual nitrite and TBARS of HTS are given in *Table 1*. The use of RE/GTE treatment caused an important reduction in the count of LAB in HTS ($P<0.05$). Micrococci and staphylococci were also affected by RE/GTE treatment, but no significant difference was observed between the control and RG1 group *(Table 1)*. On the other hand, Enterobacteriaceae were determined to be <2 log cfu/g for all treatments (data not shown).

In all treatments, the mean pH value was under 5.0. The pH value is an important factor to stabilize the HTS and ensure its safety [1]. The use of RE/GTE caused a significant change in pH in HTS. The lowest mean pH value was found RG3 treatment *(Table 1)*. In the present study, the use of RE/GTE had no significant effect on a_w, and a_w values ranged between 0.931 and 0.934 *(Table 1)*.

The use of RE/GTE caused a decrease in the residual nitrite level and the lowest value was observed in RG3 (0.3%) *(Table 1)*. In addition, the use of RE/GTE in HTS had a significant effect on the TBARS value ($P<0.01$).

Table 1. *The effects of using rosemary extract/green tea extract at the different levels on lactic acid bacteria, Micrococcus/Staphylococcus, pH, a_w, residual nitrite and TBARS of HTS (mean ± SD)*

Treatment	Lactic Acid Bacteria (log cfu/g)	*Micrococcus/ Staphylococcus* (log cfu/g)	pH	a_w	Residual Nitrite (mg/kg)	TBARS (mg MDA/kg)
Control	4.20±0.51[a]	4.81±0.29[a]	4.97±0.06[a]	0.933±0.003[a]	18.40±1.20[a]	0.83±0.05[a]
RG1	3.35±0.12[b]	4.59±0.25[ab]	4.92±0.01[ab]	0.934±0.003[a]	15.47±1.50[b]	0.75±0.02[a]
RG2	3.28±0.07[b]	4.46±0.29[b]	4.88±0.02[b]	0.931±0.003[a]	15.07±2.20[b]	0.59±0.07[b]
RG3	3.08±0.08[b]	4.28±0.37[b]	4.80±0.05[c]	0.932±0.003[a]	12.60±0.87[c]	0.62±0.03[b]
P-value	<0.01	<0.05	<0.01	>0.05	<0.01	<0.01

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-c:*** *Means with different letters in the same column are statistically different (P<0.05)*

However, no significant difference in TBARS value was observed between RG1 treatment (0.1%) and control group. RG2 and RG3 treatments gave lower TBARS values than control and RG1 treatments *(Table 1)*.

The effect of using rosemary extract/green tea extract at the different levels on enstrumental color values of HTS (mean ± SD) is given in *Table 2*. The L* and a* values of HTS were not affected by the addition of RE/GTE (P>0.05). On the other hand, RE/GTE had a very significant effect on the b* value (P<0.01). The addition of RE/GTE to the sausage batter had a very significant effect (P<0.01) on taste, odor and overall acceptability, but not on color and texture (P>0.05) *(Table 3)*. The lowest odor score was observed in the RG3 treatment. The lowest values in terms of taste and overal acceptibility were also determined in this treatment *(Table 3)*.

The effect of using RE/GTE at the different levels on volatile compounds of heat treated sucuk (mean ± SD) is given in *Table 4*. A total of 48 compounds including 1 alcohol, 7 suphur compounds, 1 acid, 2 aldehydes, 4 aliphatic hydrocarbons, 5 aromatic hydrocarbons, 2 esters, 3 ketones, 1 furan and 22 terpens were identified *(Table 4)*. In the present study, it was also observed that terpenes were the most aboundant volatile compounds in HTS. RE/GTE had a very significant effect on 3-carene and D-limonene (P<0.01). Hexanal, o-cymene, γ-terpinene, linalol and 4-terpineol were affected by the addition of RE/GTE at level of P<0.05 *(Table 4)*.

Table 2. *The effect of using rosemary extract/green tea extract at the different levels on enstrumental color values of HTS (mean ± SD)*

Treatment	Enstrumental Color Values		
	L*	a*	b*
Control	47.38±0.21[a]	17.73±0.18[a]	15.33±0.24[b]
RG1	47.01±0.10[a]	16.98±0.65[a]	15.75±0.38[b]
RG2	46.97±0.17[a]	17.17±0.40[a]	16.65±0.71[a]
RG3	46.68±0.25[a]	16.88±0.21[a]	16.76±0.40[a]
P-value	>0.05	>0.05	<0.01

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-b:*** *Means marked with different letters in the same column are statistically different (P<0.05)*

Table 3. *The effect of using rosemary extract/green tea extract at the different levels on sensory parameters of HTS (mean ± SD)*

Treatment	Color	Texture	Odor	Taste	Overall Acceptability
Control	7.70±0.30[a]	7.00±0.10[a]	7.43±0.15[b]	7.40±0.20[a]	7.43±0.15[a]
RG1	7.47±0.45[a]	7.30±0.40[a]	7.90±0.10[a]	7.57±0.25[a]	7.80±0.20[a]
RG2	7.33±0.47[a]	7.53±0.32[a]	7.77±0.23[ab]	7.20±0.20[a]	7.53±0.25[a]
RG3	7.07±0.31[a]	7.07±0.21[a]	6.73±0.21[c]	6.63±0.15[b]	6.53±0.25[b]
P value	>0.05	>0.05	<0.01	<0.01	<0.01

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-c:*** *Means with different letters in the same column are statistically different (P<0.05)*

Table 4. *The effect of using rosemary extract/green tea extract at the different levels on volatile compounds of HTS (mean ± SD) (Arbitrary Units x10⁶)*

No*	Compounds	RI	KI	Control	RG1	RG2	RG3
1	Ethanol	a	539	19.77±2.43	14.37±4.71	43.95±29.44	38.61±7.94
2	Ally mercaptan	b	610	124.18±2.57	136.80±20.09	130.80±10.79	123.70±3.75
3	Acetic acid	a	717	6.30±0.97	3.26±0.74	3.50±1.26	2.16±1.15
4	Ally methyl sullfide	b	730	11.29±7.75	13.84±5.66	7.96±3.82	10.31±2.29
5	1-(methylthio)-1-propene	b	753	1.54±2.17	2.66±3.76	2.50±3.53	2.23±2.54
6	3-Hydroxy-2-butanone	b	779	2.42±0.87	3.96±0.35	2.14±0.83	2.99±2.18
7	Toluene	a	785	1.82±2.56	2.52±3.50	2.06±0.11	1.96±0.20
8	Hexanal	a	835	3.67±0.11^{a}	3.11±0.06^{b}	3.02±0.18^{b}	2.92±0.23^{b}
9	3,3'-thiobis-1-propene	b	888	25.40±1.30	31.93±5.33	19.62±6.85	22.43±4.17
10	p-Xylene	b	892	1.43±0.61	2.81±0.34	2.04±0.48	2.07±1.17
11	Styrene	b	916	1.56±0.50	2.48±1.89	1.27±0.89	1.93±0.49
12	2-Heptanone	b	931	1.06±1.49	1.54±2.18	1.08±0.11	1.69±2.39
13	α-Thujene	b	934	2.08±1.28	2.23±3.15	2.38±0.88	5.60±1.05
14	α-Pinene	a	939	3.62±1.07	7.88±0.37	8.56±7.59	7.73±1.03
15	Methyl 2-propenyl disulfide	b	946	8.64±1.35	9.48±5.52	8.51±4.70	9.98±0.37
16	β-Pinene	b	987	8.61±0.55	11.12±1.38	10.6±1.03	9.63±1.07
17	Decane	a	1000	0.51±0.04	0.98±0.04	0.79±0.23	0.81±0.08
18	β-Myrcene	b	1005	15.58±3.28	28.38±1.09	23.39±9.91	32.39±14.56
19	2-Pentyl-furan	b	1021	0.68±0.30	0.71±0.23	0.86±0.12	0.76±0.24
20	α-Phellandrene	b	1022	4.91±0.99	12.63±4.94	14.69±0.38	24.63±8.01
21	3-Carene	b	1026	8.65±1.48^{c}	14.94±0.88^{b}	15.50±2.47^{ab}	17.56±1.54^{a}
22	2,3-Octanedione	b	1027	0.55±0.77	1.98±1.22	0.32±0.45	1.12±0.15
23	α-Terpinene	a	1030	2.57±0.63	5.17±0.07	5.16±3.34	6.06±0.52
24	D-Limonene	a	1043	17.92±0.89^{c}	34.27±7.30^{b}	39.58±5.24^{ab}	43.68±4.89^{a}
25	1-Methyl-2-(1-ethyl)-benzene	b	1046	80.29±21.68	81.86±23.99	76.79±20.89	89.13±1.89
26	Eucalyptol	b	1054	2.22±0.26	3.55±0.52	3.42±0.46	3.31±0.11
27	o-Cymene	b	1059	0.81±0.22^{b}	2.11±0.01^{a}	2.34±0.40^{a}	2.29±0.25^{a}
28	γ-Terpinene	b	1071	48.35±9.16^{c}	89.01±13.35^{b}	94.46±0.28^{ab}	113.94±18.07^{a}
29	Terpinolene	b	1095	0.87±0.13	0.92±0.08	0.95±0.06	1.38±0.46
30	4-Carene	b	1097	0.84±0.08	2.47±0.45	2.40±0.62	2.46±0.44
31	Dially disulphide	a	1116	57.73±0.57	72.91±2.74	62.40±28.04	61.56±4.91
32	2-Propenyl propyl disulfide	a	1126	3.12±0.14	4.68±0.57	3.65±3.49	3.65±0.17
33	Linalol	a	1142	39.02±3.06^{b}	58.46±0.76^{a}	55.49±2.50^{a}	57.62±1.92^{a}
34	Dodecane	a	1200	0.70±0.08	1.24±0.50	0.57±0.01	0.56±0.78
35	Camphor	b	1207	0.77±0.32	1.02±0.11	1.19±0.25	1.27±0.09
36	Hexyl butanoate	a	1214	0.51±0.09	0.63±0.16	0.60±0.23	0.68±0.11
37	4-Terpineol	b	1220	2.74±0.02^{b}	3.60±0.40^{a}	3.63±0.28^{a}	3.71±0.16^{a}
38	α-Terpineol	b	1252	1.94±2.74	5.31±0.35	4.29±0.27	5.30±0.32
39	Tridecane	a	1300	0.76±0.10	1.49±0.18	1.50±0.16	1.45±0.51
40	2-Methyl-3-phenyl- propanal	b	1318	119.46±4.90	120.26±32.20	110.32±8.11	136.21±21.38
41	α-Thujenal	b	1370	10.03±1.54	9.98±2.51	7.96±3.44	9.24±1.62
42	Tetradecane	a	1400	1.84±0.54	3.35±0.16	1.46±0.45	2.79±0.61
43	α-Cubebene	c	1408	1.34±0.25	0.85±1.20	1.27±0.68	1.63±0.03
44	Hexyl hexanoate	c	1416	1.49±0.18	0.00±0.00	1.08±0.25	0.55±0.78
45	Eugenol	b	1436	1.66±0.01	2.05±0.84	1.61±0.47	1.95±0.20
46	Isocaryophylene	c	1147	1.84±0.07	2.10±0.68	1.63±0.66	1.03±1.45
47	1,2-Dimethoxy-4-(2-propenyl)-benzene	c	1457	8.78±1.72	8.49±2.28	7.25±2.27	4.57±6.46
48	Caryophyllene	b	1490	6.16±0.91	7.94±1.35	7.96±3.01	6.05±4.51

HTS: *Heat treated sucuk,* ***KI:*** *Kovats index calculated for DB-624 capillary column installed on GC/MS,* ***RI:*** *reliability of identification,* ***a:*** *mass spectrum and retention time identical with an authentic sample;* ***b:*** *mass spectrum and Kovats index from literature in accordance;* ***a-c:*** *Means with different letters in the same row are statistically different (P<0.05)*

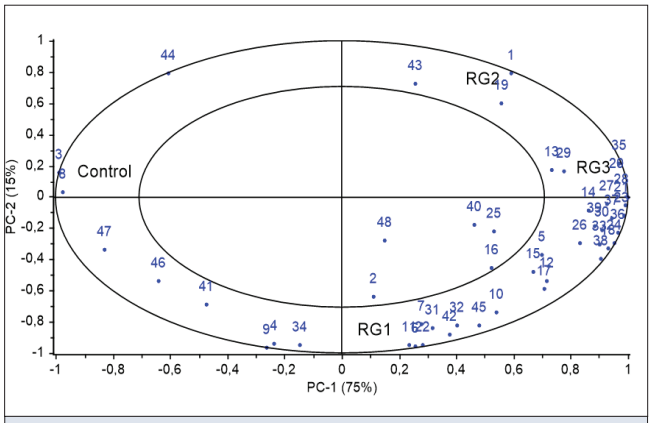

Fig 1. The principal component analysis of the relationships between factors RE/GTE treatments and volatile compounds (RG1: 0.1% RE/GTE, RG2: 0.2% RE/GTE, RG3: 0.3% RE/GTE, the numbers indicate volatile compounds in *Table 4*)

In the study, principal component analysis (PCA) was applied to assess the relationships between the treatments and volatile compounds (*Fig. 1*). The first principal component provided 75% of the total variance and separated all treatments containing RE/GTE well from the control group. In addition, most of the volatile compounds were located on the positive side of PC1 as in the RE/GTE groups. Control was found to be closely correlated with few volatile compounds while on the negative side of PC1 (*Fig. 1*).

NDMA, NDEA, NPYR and NPIP were determineted in HTS samples. NMEA, NDPA and NDBA were not dedected in any of HTS samples. The effect of using rosemary extract/green tea extract at the different levels on NDMA, NDEA, NPYR and NPIP levels of HTS is given in *Table 5*.

Table 5. *The effect of using rosemary extract/green tea extract at the different levels on NDMA, NDEA, NPYR and NPIP levels of HTS (mean ± SD) (µg/kg)*

Treatment (T)	**NDMA**	**NDEA**	**NPYR**	**NPIP**
Control	2.01±0.86[a]	1.51±0.76[a]	0.81±0.24[a]	1.05±0.41[a]
RG1	2.22±0.67[a]	1.39±0.82[a]	0.74±0.21[a]	1.00±0.26[a]
RG2	1.95±0.65[a]	1.27±0.68[a]	0.69±0.20[a]	0.98±0.24[a]
RG3	1.97±0.60[a]	1.24±0.75[a]	0.66±0.22[a]	0.92±0.25[a]
P value	> 0.05	> 0.05	> 0.05	>0.05
Cooking time (min) (CT)				
0	1.44±0.40[c]	0.78±0.26[c]	0.58±0.15[c]	0.79±0.19[b]
1	1.97±0.42[b]	1.27±0.35[b]	0.74±0.16[b]	0.94±0.16[b]
3	2.71±0.51[a]	2.01±0.81[a]	0.87±0.24[a]	1.23±0.32[a]
P-value	<0.01	<0.01	<0.01	<0.01
The interaction of T x CT	ns	ns	ns	ns

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-c:*** *Means with different letters in the same column are statistically different (P<0.05)*

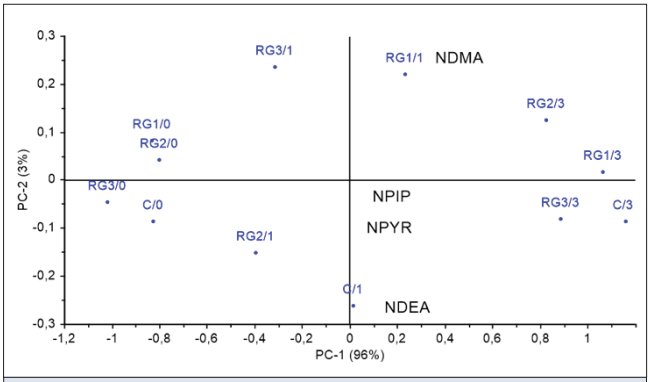

Fig 2. The principal component analysis of the relationships between RE/GTE treatments, cooking times and nitrosamines (The first number indicates the group, and the last number indicates the cooking time [min])

The principal component analysis of the relationships between RE/GTE treatments, cooking times and nitrosamines is given in *Fig. 2*. PC1 accounted for 96% of the total variance, while PC2 accounted for 3%.

Discussion

In HTS produced by subjecting to rapid ripening, LAB are important bacteria in terms of both product safety and the development of sensory properties. In industry, this product is produced using starter culture. LAB, which growth up to 10^8 cfu/g in fermentation, undergoes a certain reduction in heat treatment [4,27,31,32]. In this current study, a reduction in LAB count was also observed. However, in treatments containing RE/GTE (RG1, RG2 and RG3), the LAB count in the final product was found to be lower than in the control. In RG2 and RG3 treatments, *Micrococcus/Staphyloccus* count also decreased *(Table 1)*. It is thought that these results are probably due to the antimicrobial activity of RE/GTE. Indeed, it was reported that rosemary extract contains antimicrobial compounds such as carnosic acid, carnosol and rosmarinic acid, and green tea contains antimicrobial compounds such as epigallocatechin gallate and (-)-epicatechin gallate [33]. On the other hand, in this study, it is thought that the determination of Enterobacteriaceae number below the detectable limit is due to the drop in pH during fermentation and also the application of heat treatment [27,31].

Latilactobacillus sakei S15 strain used as a starter culture produces lactic acid during fermentation and thus the pH decreases. In this product, the pH decreases during fermentation and the subsequent heat treatment leads to an increase in the pH value, albeit slightly [31]. In the present study, the lowest pH value was observed in RG3 treatment. In a preliminary test for this study, the pH value of RE/GTE used in this study was determined as 4.91±0.01. RE/GTE lowers the initial pH value of the heat-treated sausage batter, and in this case leads to a lower pH in the final product, especially at the level of 0.3%. Jin et al.[34] reported also that rosemary extract lowers the pH value in an emulsified meat product. Similarly, Lara et al.[35] stated that rosemary extract decreased the pH value in pork patties and stated that this result was due to the fact that the active compound (carsonic acid) in this extract was an acid. On the other hand, it was reported that green tea extract did not have a significant effect on pH value in dry fermented sausages such as sucuk [20] and pepperoni [36]. Fermented sausages are environments where complex reactions take place, and their pH varies depending on various factors such as initial pH value, ingredients, buffering capacity of meat, rate and degree of acid formation of lactic acid bacteria [1,37]. On the other hand, the a_w value is a significant hurdle effect for fermented sausage. In the present study, a_w value was below 0.940, and this parameter was not affected by RE/GTE *(Table 1)*. Jin et al.[34] also stated that rosemary and thyme extracts were not effective on the a_w value of the sausages during cold storage.

HTS is a type of semi-dry fermented sausage cured with nitrite. Nitrite up to 150 mg/kg level can be used in the production of this product. Since HTS is generally consumed by cooking, the residual nitrite level draws attention as an important factor in terms of nitrosamines. In fermented sausages, the decrease in pH increases the reduction of nitrite to nitric oxide, thus reducing the amount of residual nitrite [8,27]. In this study, pH value was found below 5.0 in all groups. A similar result was observed by Sallan et al.[10] in HTS with starter culture. The low residual nitrite level in groups containing RE/GTE is thought to be due to the fact that these extracts contain antioxidant compounds [24]. In addition, the fact that the extract causes a decrease in pH also contributes to nitrite reduction [34]. In addition, in a study conducted on pepperoni, it was reported that green tea extract as a reducing agent accelerated the breakdown of nitrite [36]. Similarly, plant polyphenols (green tea or grape seed) and ascorbic acid have been found to significantly reduce residual nitrite in dry-cured sausage, and ascorbic acid is more effective [22]. On the other hand, the green tea polyphenols decreased the residual nitrite content in dry cured bacon faster than other antioxidants (grape seed extract and alpha tocopherol) [23]. However, it is emphasized that nitrite reduction depends on various factors such as the pH of the meat, ingoing nitrite level, processing and storage conditions, presence of reducing agents, and type of raw meat [23].

According to the TBARS results, the addition of 0.2% RE/GTE to the HTS batter caused sufficient to retard lipid oxidation. However, TBARS value was found below 1 mg MDA/kg even in the control group. Lin et al.[36] also found that green tea extract inhibited lipid oxidation in pepperoni. A similar result was also found in sucuk by Bozkurt [20]. Jongberg et al.[18] reported that green tea and rosemary extracts decreased the TBARS value in bologna type sausages. In another study on dry cured sausage, it was determined that green tea polyphenol was more effective in inhibiting lipid oxidation than ascorbic acid and grape seed polyphenol [22]. In addition, green tea polyphenol has been reported to have the most potent antioxidant activity in dry cured bacon production [23]. Antioxidant compounds from rosemary and green tea inhibite lipid oxidation by functioning either as free-radical scavengers or metal chelators [36,38]. Carnosic acid and carnosol components play an important role in the antioxidant activity of rosemary extract [39]. In green tea, on the other hand, catechins are active [40].

In present study, the use of RE/GTE had no significant

effect on L* and a* values *(Table 2)*. Similarly, in the studies carried out on pepperoni [36] and sucuk [20], it was reported that green tea extract did not affect the color parameters (L*, a* and b* values). Also, in a study conducted on cured pork sausage with white kimchi powder, it was reported that gree tea, rosemary and their combination did not affect the L* value compared to the control group [38]. RG2 and RG3 treatments showed higher b* values than control and RG1 *(Table 2)*. Similarly, it was stated that rosemary and green tea increased the b value in pork sausage and this result was due to the inhert pigments of plant-derived extracts [38]. Also, Jin et al.[34] reported that the use of rosemary extract in emulsified sausage increased the b* value.

In this study, the use of RG/GTE reduced the overall acceptability score. But, none of the values determined in the RG3 treatment were below 6.0. In fact, it was indicated that rosemary and green tea extracts are additives that have a positive effect on the taste and appearance of meat products, and that the effect of green tea extract on the flavor of the final product is limited [40].

HTS is a product made by applying a short-term fermentation, heat treatment and drying stages. Due to the short production time and heat treatment, reactions such as lipid oxidation and proteolysis occur to a limited extent. Therefore, terpenes have an important share in the aromatic profile of the product. The main source of terpenes is spices [4,31]. Hexanal is described as the major oxidation product, and sourced from oxidation of n-6 fatty acids such as linoleic and archidonic acids. Its high concentrations indicator flavor deterioration in meat products often resulting in a rancid aroma [30]. In this study, the use of RE/GTE resulted in a reduction in hexanal levels. However, this decline was not dependent on an increase in the RE/GTE ratio *(Table 4)*. This result is due to the antioxidant properties of rosemary and green tea extracts. This result is due to the antioxidant properties of rosemary (carnosic acid and carnosol) [41] and green tea (mainly flavonoids) extracts [39]. The levels of 3-carene, D-limonene and γ-terpinene increased due to the addition of RE/GTE. However, no difference was observed between RG2 and RG3 in terms of these compounds *(Table 4)*. It has been reported that the level of some monoterpenes, including 3-carene, increased with the increase in the amount of rosemary extract in fresh pork sausages [33]. On the other hand, the other three compounds (o-cymene, linalol and 4-terpineol) were affected by RE/GTE addition gave the highest mean value in RG3 treatment, but did not differ statistically from RG1 and RG2 *(Table 4)*. The most important source of terpenes is spices [30]. In this study, black pepper, red pepper, allspice and cumin were used as spices in the production of HTS. PCA analysis showed that the use of RE/GTE resulted in an increase in the content of terpenes in HTS.

Nitrosodimethylamine (NDMA) is defined by the International Agency for Research on Cancer as probably human carcinogen (Group 2A) [42]. NDMA is commonly determined in fermented sausages. It has been reported that NDMA in HTS varies between 1.71 and 3.71 μg/kg [9]. NDMA levels in fermented sausages in Danish and Belgian markets were found to range from undetectable to 4 μg/kg and undetectable to 7.2 μg/kg, respectively [43]. In the present study, the mean NDMA content was determined as 1.44±0.40 μg/kg in samples without cooking. The use of RE/GTE did not show a significant effect on NDMA level *(Table 5)*. Similarly, in a study conducted to determine the effects of plant polyphenols (green tea and grape seed polyphenols) and ascorbic acid on nitrosamine level in a dry fermented sausage, no significant difference was observed between the groups in terms of NDMA at the end of ripening. In the same study, the NDMA content varied between 0.56 and 0.76 μg/kg dry matter [22]. For dry-cured bacon, after three weeks of storage, it was reported that the control group and samples containing alpha-tocopherol contained more NDMA than samples containing green tea polyphenol and green tea + grape seed extract. It was also emphasized that NDMA and sodium nitrite showed a negative correlation [23]. Nevertheless, it was determined that the use of rosemary extract, grape seed extract and green tea polyphenol reduced the NDMA content in Western style smoked sausage [24]. In the present study, unlike the RE/GTE factor, the cooking time had a significant effect on NDMA *(Table 5)*. NDMA content increased as the cooking time increased. In a previous study on HTS, it was determined that the NDMA content increased as the cooking time increased [10].

NDEA is determined less frequently in fermented products than other nitrosamines such as NDMA, NPYR and NPIP. Kaban et al.[9] and Sallan et al.[10] stated that the NDEA in the heat-treated sausage samples was below the detectable limit. However, Akansel et al.[27] found that average NDEA level in HTS samples without additional heat treatment was 0.61 μg/kg. In the present study, the mean NDEA content was found to be 0.78±0.26 μg/kg for samples without cooking *(Table 5)*. As can be seen from *Table 5*, the NDEA level decreased as the RE/GTE ratio increased. However, the differences between treatments were not statistically significant. The interaction of RE/GTE and cooking time also had no significant effect on NDEA *(Table 5)*. In contrast, The NDEA content increased with the progression of cooking time. Similar results were also reported by Akansel et al.[27]. On the other hand, Li et al.[22] reported that NDEA was detected at early ripening of dry cured sausage. In the same study, it was stated that there was no significant difference in terms of NDEA between the control group and green tea polyphenol treatment on the 7th day of ripening [22].

NPYR, among the possibly (Group 2B) carcinogenic compounds [42], is an important nitrosamine for cured meat products. The level of this nitrosamine in HTS ranges from 1.65 to 7.29 µg/kg [9]. The maximum NPYR level found in salami from Belgium was 6.9 µg/kg [43]. In the present study, it was observed that the NPYR level decreased depending on the increase in the RE/GTE ratio. However, as can be seen from *Table 5*, the differences between the treatments were not found to be statistically significant. On the other hand, Li et al.[22] stated that the control group had a higher NPYR value than the groups containing green tea or grape seed polyphenols at the end of the 28 day ripening in a dry sausage. While many spices are good sources of NPYR, pyrrolidine is the major source of this nitrosamine, and putrescine is also stated to be effective in the formation of NPYR [8,15]. On the other hand, it was reported increase of ingoing nitrite level in HTS unaffected the NPYR level [10]. In the present study, cooking time had a significant effect on NPYR. In contrast, there was no relationship between RE/GTE and cooking time *(Table 5)*. Cooking is an important factor for this nitrosamine [10].

Another important nitrosamine found in fermented sausages is NPIP. Piperine and piperidine in black pepper play an important role in the formation of this compound [27]. Black pepper is also an important spice for HTS. In the Turkish market, the NPIP level in this product can reach high levels depending on the level of black pepper [9]. In this study, NPIP was not affected by the use of RE/GTE. However, cooking time had a very significant effect on this nitrosamine *(Table 5)*. Sallan et al.[10] and Yılmaz Oral [44] and Yılmaz Oral [45] reported that cooking degree was an important factor and an increase in cooking time resulted in increased NPIP. While fermented sausages are consumed raw in many countries, sucuk and HTS are generally consumed after cooking processes such as frying, barbecue and roasting in Türkiye. Therefore, cooking time or cooking degree is of great importance for reducing the risk of nitrosamines [45,46].

According to the result of PCA, PC1 provided good discrimination between treatments with different cooking times. While, all treatments of the 3 min cooked samples were placed on the positive side of PC1, the uncooked samples (0 min) were on the negative side of PC1. However, the application of 1 min cooking time differed between the treatments. Control and RG1 treatments showed a higher correlation with nitrosamines, placing on the positive side of PC1. In contrast, samples of RG2 and RG3 cooked for 1 min were on the negative side of PC1 and showed a weaker correlation with nitrosamines. It was determined in many studies that the cooking process/time is an important factor in the formation of nitrosamines.

In conclusion, the use of RE/GTE at different levels in the production of HTS, a type of semi-dry fermented sausage, caused decreases in both the pH value and the technologically important LAB and M/S numbers in the final product. However, these decreases did not occur in a way that would negatively affect product properties. RE/GTE did not cause any change in a_w value. Since the RE/GTE utilization rate was 0.2% and 0.3%, the TBARS value decreased, indicating that this extract combination slowed down lipid oxidation in HTS. While RE/GTE had no effect on the L* and a* values, it increased the b* value by 0.2% and 0.3%. In addition, due to the reducing property of the extract, it reduced the residual nitrite level, which is an important result in terms of nitrosamine formation. Sensory parameters of color and texture were not affected. A RE/GTE level of 0.3% resulted in significant reductions in both odor, taste and overall acceptability parameters. The use of RE/GTE had a limited effect on volatile compounds. It decreased hexanal level, and increased six terpenes. On the other hand, NDEA, NPYR and NPIP levels decreased as the RE/GTE ratio increased, but these decreases were not statistically significant. NDMA was also not affected by the use of this extract mixture. As the cooking time progressed, the levels of the determined nitrosamines increased. However, the use of the extract did not cause a different effect during cooking. Considering all these results, it was concluded that the RE/GTE mixture can be used up to 0.2% level and that the heat-treated sucuk should not be subjected to any additional cooking before consumption, even though the total nitrosamine level is below 10 ug/kg even in products cooked for 3 min.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

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Ethical Statement

Ethics approval was not required for this research due to conducting *in vitro* in the laboratory.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contribution

ZFYO: Conceptualization, methodology, validation, formal analysis, investigation, writing original draft. GK: Conceptualization, methodology, writing-review and editing. Final approval of the completed article: ZFYO and GK.

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