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Effect of Biofermentation with Taxifolin on Physicochemical and Microbiological Parameters of Cold-Smoked Pork Sausages

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SUMMARY

The aim of this work was to evaluate the effect of taxifolin in different commercial recipes: with *Leuconostoc carnosum* and with a mixture of strains *Pediococcus pentosaceus* and *Staphylococcus xylosus*. Ultra Performance Liquid Chromatography (UPLC) analysis demonstrated that after 181 days of storage total taxifolin content was the highest in samples with taxifolin and *L. carnosum* (60 %), compared to the first day of storage. The sausages with taxifolin and the mixture of *P. pentosaceus* and *S. xylosus* (56 %) followed next. Taxifolin contributed improving the hygienic quality of sausages without significant effect on the growth of lactic acid bacteria. The accumulation of biogenic amines, including histamine and putrescine, was more effectively reduced in sausages inoculated with the taxifolin and *P. pentosaceus* and *S. xylosus* mixture. Using this mixture, the rate of lipolysis and processes of lipid oxidation were effectively slowed down. Samples with taxifolin + *L. carnosum* showed the highest free radical scavenging activity on the first day of the study ((77.37±1.31) %) ($p<0.05$ in all samples). Mixtures containing taxifolin and starter cultures better bind free radicals than taxifolin alone. The colour parameters (*L*, a*, b**) of preparations and final

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products were significantly influenced by the used taxifolin and starter cultures and storage time ($p<0.05$ in all samples).

Key words: *Leuconostoc carnosum, Pediococcus pentosaceus, Staphylococcus xylosus*, biogenic amines

INTRODUCTION

The application of bioflavonoids as natural antioxidants for treatment of cold-smoked pork sausages with commercial recipes has not been discussed in the available literature. Taxifolin (also known as 3,5,7,3′,4′-pentahydroxy-flavanone or 2,3-dihydroquercetin) is a member of the group of flavanones (*1*). The satisfactorily pure taxifolin may be extracted from Siberian larch (*Larix sibirica* Ledeb.) and also is abundant in citrus fruits, grapes, olive oil, and onions (*2,3*). Being a common bioactive constituent of foods and herbs, taxifolin has been shown to exert a wide range of positive biochemical and pharmacological effects on human health. As a typical flavonoid, it inhibits free radical formation (*4*), influences the physical properties of lipids in biological membranes (*5*), has anti-inflammatory and analgesic properties (*6*), as well as cardioprotective, and neuroprotective effects (*7,8*).

Taxifolin has many health-promoting effects, but is unstable to the light, pH and thermal treatment (*9-11*). Besides, little is known about the degradation behaviour of taxifolin throughout the storage time. Knowledge about the stability of taxifolin in food processing is important in order to predict the duration of physiological effects in food and beverages (*12*). The food additives authorized for use in Europe are reported in European Regulation (EC) No 1129/2011 (*13*), as well as the levels of their maximum permitted usage in the food where their use is permitted. On 13 December 2016, the EFSA NDA Panel adopted the Scientific Opinion on the safety of taxifolin - as a novel food ingredient in non-alcoholic beverages, yogurts, chocolate confectionery and food supplements pursuant to Regulation (EC) No 258/971 (*14*). The maximum use levels proposed by the applicant were 0.02 g/L for non-alcoholic beverages, 0.02 g/kg for yogurts and 0.07 g/kg for chocolate confectionery. The maximum proposed daily intake of taxifolin from food supplements was 100 mg/day (*15*) although scientific literature indicates the most suitable concentration of taxifolin in meat products to be between 0.006 and 0.04 %, depending on the fat content (*16*).

In the future, taxifolin could be used as a natural antioxidant and antimicrobial additive in the food industry (*17*), such as cold-smoked fermented sausages with starter cultures due to the fact that taxifolin has been described as having antimicrobial (*18*) and radical

scavenging activity (*19*), and a protective role in plants against pathogens (*20*). The addition of selected starter cultures has been reported to improve the safety of fermented sausages by restraining the development of undesired microorganisms, thus reducing the risk of pathogenic and spoilage bacteria, maintaining stability and shelf life, and enhancing the sensory characteristics of the meat products (*21*).

The aim of this work was to evaluate the effect of mixtures of taxifolin combined with different commercial recipes on the physicochemical and microbiological parameters in order to select the most suitable mixture for the safety and quality of cold-smoked pork sausages during storage.

MATERIALS AND METHODS

Preparation of taxifolin solution

Taxifolin (≥85 %) obtained from Sigma-Aldrich GmbH (Buchs, Switzerland). Taxifolin was dissolved in several drops of ethanol (96 %) and diluted with double distillated water and added to the minced pork (0.517 mg/kg).

Sausage production and sampling procedures

Eight different batches of pork sausage were manufactured using different techniques: three batches with the addition of different starter cultures in a proportion defined by the manufacturer, three batches with the addition of starter cultures and taxifolin, one batch with only taxifolin and one control batch without starter culture or taxifolin. The batches were prepared as follows: CR-1 with starter culture of *Leuconostoc carnosum*, CR-2 with *Pediococcus pentosaceus* and *Staphylococcus xylosus*, CR-3 with *P. pentosaceus* in high quantity and *S. xylosus*, CR-4 with taxifolin and *L. carnosum*, CR-5 with taxifolin, *P. pentosaceus* and *S. xylosus*, CR-6 with taxifolin, *P. pentosaceus* in high quantity and *S. xylosus*, CR-7 with only taxifolin, and CR-8 (control) without taxifolin or starter culture.

The contents of pork sausages were as follows: whole pork muscle and back fat cuttings (80 %), raw pork ham (20 %) was purchased from a local establishment in Kedainiai, Lithuania. Sodium chloride (25 g/kg), lactose (20 g/kg), dextrin (20 g/kg), sodium caseinate (20 g/kg), glucose (7 g/kg), black pepper (1.5 g/kg), white pepper (1 g/kg), sodium ascorbate (0.5 g/kg) was purchased from company Sirmulis, Lithuania. The whole muscle cuts and raw ham were minced through a 13 mm diameter mincing plate and mixed together with the other ingredients for 3 min. The mix was kept at 4 ºC for 24 h in a refrigerator (Snaigė FR240-1101AA, Kaunas, Lithuania) and then stuffed into natural casings with a diameter of 45 mm and a length of 9 cm. Thin cold smoke was applied (Helia Smoker, Germany) over a period

of 24 h until the casings developed yellow to light brown colour. The sausages were fermented for 2 days at 15 ºC, and 85 % of relative humidity, then transferred into a drying ripening chamber where they were kept for 18 days at 10–12 ºC, and 75–80 % relative humidity until the yield was 87 % compared to the original meat weigh. The cold-smoked sausages were then kept for 181 days at 15 ºC, and 75 % relative humidity.

Taxifolin stability, physicochemical parameters and microbiological profile of sausages were analysed after 1, 33, 128 and 181 days of storage. The analyses were carried out in triplicates, and experiments were repeated twice.

Determination of taxifolin in cold-smoked sausages using Ultra Performance Liquid Chromatography

All the reagents and standards were of analytical grade, HPLC-grade acetonitrile, trifluoroacetic acid, taxifolin from Sigma-Aldrich GmbH (Buchs, Switzerland). Deionized water was acquired from a Milli-Q purification system (Millipore Corporation, Bedford, MA, USA).

Sausage samples (1 g) were transferred into a 100 mL stoppered conical flask. Ethanol (96 % 100 mL) was added, and placed on a rotary shaker (Thermo Scientific MaxQ 4000 shaker, Waltham, MA, USA) under agitation (300 rpm) for 30 min, then the mixture was filtered through filter paper (Whatman, 601 A, Maidstone, UK). The clear portion was kept in a freezer (Snaigė FR240-1101AA, Kaunas, Lithuania) for at least 20 min for the extraction of fat. The mixture was then centrifuged at 7500x*g* for 20 min at 4 °C (TJ-6 refrigerated centrifuge; Beckman Instruments, Palo Alto, CA, USA). The upper phase was filtered through a 0.2 µm pore-size syringe filter (Acrodisc LC13 PVDF, Sigma-Aldrich, ON, Canada) and injected into the HPLC unit.

Chromatographic analysis was carried out with a Waters Acquity UPLC system consisting of binary solvent manager, auto sampler, column manager, and PDA detector (Milford, MA, USA). The UPLC column was a 2.1 mm× 100.0 mm Acquits UPLC C18 BEH (Waters) with 1.7 µm particles. The mobile phase consisted of 0.1 % taxifolin in deionized water (A) and acetonitrile (B). The gradient was formed as follows: initially, the separation was started with 88 % A. It was kept at this concentration for 1 min, and then in 3 min A was decreased to 70 %, and from there within 3 min to 10 %, and was held at 10 % for 1 min. After that the column was allowed to equilibrate for 2 minutes. The flow rate was 0.5 mL/min, and the injection volume was 1 µL. The detector was set in the 200–400 nm range. The chromatographic data was acquired and processed with Empower 3 software (Milford, USA) (*22*).

Chemical analyses

The moisture content of cold-smoked sausage samples was obtained according to ISO 1442:2000 (*23*). The pH of sausages was measured according to the standard method for determination of meat pH: EN ISO 2917:2002 (*24*). pH measurements were carried out using a PP-15 pH-meter (Sartorius Professional meter for pH Measurement, Germany).

For radical scavenging activity, the method used by Takao *et al.* (*25*) was adopted with suitable modifications from Kumarasamy *et al*. (*26*). The diluted sample (200 µL) was mixed with 800 µL of tris-HCl buffer (100 mM, pH 7.4). To this was added 1 mL of 500 µM DPPH in ethanol and the mixture was vortexed and absorbance measured at 517 nm after 20 min incubation in the dark. Percent radical scavenging activity was calculated as:

[(Control absorbance - Extract absorbance) / (Control absorbance)] x 100 /1/

Acid value of the extracted lipids was determined according to EN ISO 660:2009-10 (*27*). One gram extracted lipids were dissolved with 50 mL neutral solvent solution (diethyl ether (50 mL), ethyl alcohol (50 mL), and 1 mL 1% phenolphthalein solution). The titration was carried out with 0.1 mol/L NaOH at constant shaking until the formed pink color was persisted for 15 s.

Peroxide value of the studied lipids is determined according to EN ISO 3960:2010 (*28*) iodometric method, and was expressed in mmolO2/kg lipids. About 3.0 g fat was mixed with 50 mL of the solvent mixture (glacial acetic acid: chloroform 3:2), 1mL freshly prepared saturated potassium iodide solution, and 100 mL water. The titration was carried out with 0.01 mol/L sodium thiosulfate solution, using 1 mL starch solution and 0.1g of Thyodene indicator until the blue colour disappears.

Microbial analysis

Samples of 10 g were taken at random for each batch, and aseptically weighed into a sterile stomacher bag with 90 mL of sterile buffered 0.1 % (m/V) peptone water (Liofilchem, Roseto degli Abruzzi, Italy) and homogenized for 1 min in a model 400 Stomacher (Seward Medical, London, UK). Serial decimal dilutions were made, and lactic acid bacteria were determined by plate count on de Man, Rogosa and Sharpe agar (MRS, Oxoid, Hampshire, UK) after incubation at 30 ºC for 120 h. The count of yeast and mould colonies were determined by plate count on dichloran rose chloramphenicol (DRBC) agar (Sigma-Aldrich, Abruzzi, Italy) after an incubation at 25 ºC for 120 h, and total count of mesophilic bacteria was determined on plate count agar (PCA, Liofilchem) after an incubation at 30 ºC for 72 h in

a thermostat. After incubation, colonies were counted according to ISO 7218/2007 (*29*). The microbiological data was transformed into logarithm of the number of colony forming units (CFU/g).

Biogenic amine content

A reversed-phase high-performance liquid chromatography (RP-HPLC) method was used for the quantitative analysis of biogenic amines tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine. The whole cured sausage (edible parts) was catted into small pieces and mashed mechanically using a homogeniser (Moulinex Masterchef 20, Nieune, France). Biogenic amine content was extracted from a homogenized sample with 0.4 mol/L perchloric acid (Sigma-Aldrich, Abruzzi, Italy). The derivatization of samples was carried out using the modified method of Ben-Gigirey *et al.* (*30*). The extract was derivatized for 45 min with dansyl chloride (5-dimethylaminonaphtalene-1-sulfonylchloride) (Sigma-Aldrich, Abruzzi, Italy) solution in acetone (Sigma-Aldrich, Abruzzi, Italy) at 40 °C. The samples were filtered through a 0.45 μm membrane filter (Millipore), and 10 μL of the sample were injected into chromatographic system (Agilent 1200 series; Waldbronn, Germany). The analysis was performed using LiChro column CART® 95 125-4 (Merck, Darmstadt, Germany).

Colour determination

Meat surface colour was measured, using a reflectance spectrophotometer (Minolta CM-2002; Osaka, Japan). Parameters measured in the reflection mode: *L**, *a** and *b** (corresponding to the brightness, redness and yellow coordinates according to the CIE scale) (*31*).

Statistical analysis of the data

Data were statistically analyzed using the SPSS 20.0 software (SPSS Inc., Chicago, Illinois, USA). The differences between different-date trials were evaluated by the analysis of variance method (one-way ANOVA) with a significant level of $p<0.05$ (*32*). Multiple comparisons were estimated by Fishers Least Significant Difference method, and the Dunnet test was applied when control group was present. Student's t-test was used to determine average values of indicators, standard deviations and linear correlations. The correlation was considered reliable when $p<0.05$.

RESULTS AND DISCUSSION

Acidity of cold-smoked pork sausages

During storage, starter cultures and taxifolin in cold-smoked pork sausages ensured the acidification of meat. The decrease of the active acidity (pH) was caused by the fermentation of carbohydrates, which leads to the accumulation of organic acids, mainly lactic acid, during the storage period (*33-35*). The significant difference between the control and the samples with the added starter culture was detected before day 33 of the study ($p<0.05$ in all samples) (Table 1). We found a negative correlation between pH and lactic acid bacteria ($r=-0.621$, $p<0.05$). Throughout the study, significantly lower ($p<0.05$) Ph values was found only in samples CR-3 and CR-6, compared to control (CR-8). One taxifolin or with starter cultures did not have significant effect on pH during storage. At the end of the study, the final pH value of cold-smoked pork sausages was 5 or lower. This data coincided with Berardo *et al.* (*36*), who claimed that when using the Nordic sausage production technology, the final pH of sausage is 5 or lower, while when using the Mediterranean technology, the final pH of sausage is between 5 and 6.

Table 1. Effect of taxifolin and commercial recipes on the pH in cold-smoked pork sausages during storage

Sample	*t*(storage)/day			
	1	33	128	181
	pH			
CR-1	$(4.83\pm0.02)^a$	$(4.93\pm0.02)^a$	$(5.24\pm0.02)^a$	$(5.36\pm0.01)^b$
CR-2	$(4.85\pm0.01)^a$	$(4.86\pm0.02)^a$	$(5.31\pm0.03)^a$	$(5.41\pm0.01)^a$
CR-3	$(4.72\pm0.01)^a$	$(4.82\pm0.01)^a$	$(5.17\pm0.02)^b$	$(5.23\pm0.03)^b$
CR-4	$(4.81\pm0.02)^a$	$(4.94\pm0.03)^a$	$(5.25\pm0.01)^a$	$(4.41\pm0.01)^a$
CR-5	$(5.84\pm0.02)^a$	$(4.91\pm0.02)^a$	$(5.31\pm0.03)^a$	$(5.43\pm0.02)^a$
CR-6	$(4.72\pm0.03)^a$	$(4.78\pm0.01)^a$	$(5.07\pm0.01)^b$	$(5.21\pm0.01)^b$
CR-7	$(5.01\pm0.01)^b$	$(5.11\pm0.04)^b$	$(5.34\pm0.02)^a$	$(5.49\pm0.02)^a$
CR-8	$(4.98\pm0.02)^b$	$(5.16\pm0.01)^b$	$(5.29\pm0.02)^a$	$(5.46\pm0.01)^a$

Results are expressed as mean value±standard deviation. Different letters in superscript indicate significant difference between the samples in the same row (corresponding to the same batch). CR-1=sausage sample fermented with starter culture of *Leuconostoc carnosum*, CR-2=sausage fermented with *Pediococcus pentosaceus* and *Staphylococcus xylosus*, CR-3=sausage fermented with *P. pentosaceus* in high quantity and *S. xylosus*, CR-4=sausage with taxifolin and taxifolin *L. carnosum*, CR-5=sausage with taxifolin, taxifolin*P. pentosaceus* and *S. xylosus*, CR-6=sausage with taxifolin, taxifolin*P. pentosaceus* in high quantity and *S. xylosus*, CR-7=sausage with taxifolin, and CR-8=sausage without taxifolin or starter culture (control)

Microbiological profile of cold-smoked pork sausages

Table 2 shows the microbiological profile of cold-smoked pork sausages. The use of starter cultures increased ($p<0.05$) population of lactic acid bacteria in sausages, compared to the control sample (without starter culture). Our findings are similar to those reported by Kamenik *et al.* (*37*), who observed that the grow of lactic acid bacteria occurs during the first days of fermentation and the population density peaks at 10^9 cells/g. This is especially important, because taxifolin does not inhibit lactic acid bacteria growth in the fermented sausages.

We also evaluated the yeast and mould counts. Taxifolin had an inhibitory effect on mould and yeast in CR-2 and CR-5 samples at 1-33 days, and in CR-1 and CR-4 mixtures at 33-181 days, compared to the control sample ($p<0.05$). Malterud *et al.* (*38*) studied the antimicrobial properties of flavonoids from *Salix caprea* and found that taxifolin was effective against bacteria and fungi. Taxifolin has also been identified in *Populus tremuloides* black galls, which are a type of plant tumours found in trees resistant to fungal infections (*39*).

Table 2. Effect of taxifolin and commercial recipe on lactic acid bacteria, mesophilic bacteria, yeast and moulds counts in cold-smoked pork sausages during storage

Microbiological profile	Sample	*t*(storage)/day			
		1	33	52	147
		N/(log CFU/g)			
Lactic acid bacteria	CR-1	(7.16±0.49)^{b}	(5.97±0.20)^{ab}	(6.33±0.42)^{a}	(4.65±0.34)^{a}
	CR-2	(7.02±0.33)^{b}	(6.25±0.31)^{ab}	(6.44±0.27)^{a}	(4.41±0.37)^{a}
	CR-3	(6.95±0.39)^{ab}	(6.63±0.45)^{b}	(6.65±0.36)^{a}	(5.79±0.41)^{a}
	CR-4	(7.27±0.40)^{b}	(5.23±0.37)^{ab}	(6.28±0.28)^{a}	(4.30±0.28)^{a}
	CR-5	(7.11±0.48)^{b}	(6.38±0.26)^{ab}	(6.94±0.35)^{a}	(5.00±0.40)^{a}
	CR-6	(6.33±0.28)^{a}	(6.97±0.34)^{b}	(6.87±0.22)^{a}	(6.16±0.32)^{a}
	CR-7	(6.15±0.35)^{a}	(6.05±0.29)^{ab}	(3.69±0.31)^{b}	(3.00±0.22)^{b}
	CR-8	(6.49±0.41)^{a}	(5.73±0.30)^{a}	(3.89±0.28)^{b}	(3.30±0.27)^{b}
Total count of yeasts and moulds	CR-1	(3.01±0.11)^{a}	(3.11±0.23)^{ab}	(3.20±0.31)^{b}	(3.29±0.46)^{b}
	CR-2	(2.00±0.20)^{b}	(2.68±0.18)^{a}	(3.49±0.24)^{a} ^{b}	(3.98±0.28)^{ab}
	CR-3	(2.62±0.21)^{ab}	(2.94±0.29)^{a}	(3.27±0.33)^{a} ^{b}	(3.98±0.28)^{ab}

	CR-4	(3.29±0.14)^{a}	(3.20±0.22)^{ab}	(3.17±0.27)^{b}	(3.15±0.35)^{b}
	CR-5	(2.20±0.25)^{b}	(2.71±0.34)^{a}	(3.84±0.20)^{a}	(4.15±0.44)^{a}
	CR-6	(2.85±0.19)^{ab}	(3.71±0.28)^{ab}	(3.89±0.15)^{a}	(4.08±0.31)^{ab}
	CR-7	(2.99±0.23)^{ab}	(3.94±0.17)^{b}	(3.91±0.34)^{a}	(3.95±0.29)^{a}
	CR-8	(3.92±0.31)^{a}	(4.11±0.24)^{b}	(4.27±0.44)^{a}	(4.38±0.37)^{a}
Total count of mesophilic bacteria	CR-1	(7.38±0.32)^{a}	(6.89±0.29)^{ab}	(6.34±0.34)^{a}	(4.70±0.25)^{a}
	CR-2	(7.46±0.25)^{a}	(7.03±0.47)^{ab}	(6.45±0.21)^{a}	(4.48±0.37)^{a}
	CR-3	(8.18±0.41)^{a}	(7.62±0.24)^{b}	(6.43±0.30)^{a}	(5.78±0.34)^{b}
	CR-4	(7.43±0.45)^{a}	(6.89±0.36)^{a}	(4.79±0.43)^{b}	(4.00±0.20)^{a}
	CR-5	(7.51±0.37)^{a}	(7.71±0.29)^{b}	(6.77±0.22)^{a}	(6.00±0.40)^{b}
	CR-6	(8.06±0.26)^{a}	(7.99±0.33)^{b}	(6.99±0.38)^{a}	(6.11±0.29)^{b}
	CR-7	(8.24±0.34)^{a}	(6.87±0.40)^{ab}	(6.18±0.25)^{a}	(3.00±0.10)^{a}
	CR-8	(6.94±0.21)^{a}	(6.24±0.28)^{a}	(6.35±0.41)^{a}	(3.30±0.20)^{a}

Results are expressed as mean value±standard deviation. Different letters in superscript indicate significant difference between the samples in the same row (corresponding to the same batch). Sample abbreviations are given in Table 1

Accumulation of biogenic amines in cold-smoked sausages

Biogenic amines can be detected in raw materials and food products that are formed during metabolic processes. The main biogenic amines produced in the sausage during fermentation are putrescin, cadaverine and tyramine (*40*). In our study, sausages mainly contained putrescine (51.10-86.74 mg/kg), tyramine (14.98-56.61 mg/kg), cadaverine (19.23 -34.64mg/kg) and spermine (1.13-8.76 mg/kg) (data not shown). After 33 days, CR-5 group had significantly ($p<0.05$) lower mass fraction of histamine (1.98 mg/kg) and putrescine (18.02 mg/kg) compared to other sausage groups (8.23-19.42 and 37.11-68.47 mg/kg respectively). A significantly ($p<0.05$) higher mass fraction of tyramine was observed in CR-2 batch compared to CR-5 ((35.29±3.11) and (21.56±4.30) mg/kg respectively). The total biogenic amine content in sausages inoculated with CR-5 mixture was 25 and 49 % lower ($p<0.05$) than that in CR-2 and CR-8 (control) sausages. CR-4 batch presented a 31 % lower

biogenic amine content than in CR-1, which presented the highest total biogenic amine content (204.47±11.59 mg/kg).

Stability of taxifolin in cold-smoked sausages

A far more objective view of changes in taxifolin contents in the cold-smoked sausages can be obtained after recalculation of the taxifolin content on a dry mass basis. This recalculation eliminates the effect of variable water content on changes in taxifolin content during production. After 181 days of storage, total taxifolin content was the highest in CR-4 recipe (0.027 mg/kg, followed by CR-6 recipe (0.025 mg/kg) ($p<0.05$), while the sausages treated only with taxifolin (CR-7) retained only 0.012 mg/kg of taxifolin (Table 3). UPLC analysis demonstrated that taxifolin was more stable in samples fermented with commercial starter cultures, which could be related to a stronger acidification during the first 33 days.

Table 3. Taxifolin content of cold-smoked pork sausages on dry mass basis during storage

Sample	*t*(storage)/day			
	1	33	128	181
	w(taxifolin)/(mg/kg)			
CR-4	$(0.045\pm0.004)^{a2}$	$(0.041\pm0.003)^{a2}$	$(0.033\pm0.001)^{b2}$	$(0.027\pm0.002)^{c2}$
CR-5	$(0.043\pm0.003)^{a23}$	$(0.038\pm0.002)^{a2}$	$(0.029\pm0.002)^{b2}$	$(0.022\pm0.003)^{c2}$
CR-6	$(0.041\pm0.003)^{a23}$	$(0.038\pm0.003)^{a2}$	$(0.030\pm0.001)^{b2}$	$(0.025\pm0.001)^{c2}$
CR-7	$(0.039\pm0.004)^{a3}$	$(0.030\pm0.001)^{b3}$	$(0.022\pm0.002)^{c3}$	$(0.012\pm0.001)^{d3}$

Results are expressed as mean value±standard deviation. Different letters in superscript indicate significant difference between the samples in the same row (corresponding to the same batch) and different numbers in superscript indicate significant difference between the samples in the same column (corresponding to the same storage time). Sample abbreviations are given in Table 1

Antioxidant properties of cold-smoked sausages

The effect of taxifolin on antioxidant properties of cold-smoked pork sausages during storage is shown in Table 4. CR-4 sample had the highest free radical scavenging activity ((77.37±1.31) %) on the first day of the study, followed by CR-5, CR-6 and CR-7 ((76.89±0.77), (76.68±0.68) and (73.04±0.42) %, respectively) ($p<0.05$). Mixtures containing taxifolin with starter cultures better bind free radicals than one taxifolin. On the last day of the study (181 days), the highest antioxidant activity was detected in CR-4, CR-5 and CR-6 mixtures (55.59±0.50, 51.14±0.28 and 54.48±0.43 %, respectively). For 95 % purity taxifolin, the antioxidant activity is 19.93, for 80-90 % purity – 15.16. Meanwhile, other popular antioxidants, such as luteolin, quercetin, ascorbic acid, and vitamin E have antioxidant activity values of 12.50, 10.98, 2.10, and 1.30. The antioxidative activity in taxifolin arises

from the ability of groups to donate hydrogen atoms at the developing reaction with the free-radical oxygen metabolites resonance stabilized phenol radical (*41*).

Table 4. Effect of taxifolin and commercial recipes on antioxidant activities of cold-smoked pork sausages during storage

	Sample	*t*(storage)/day			
		1	33	128	181
Scavenging activity/%	CR-1	(64.58±0.45)^{a}	(57.36±0.77)^{a}	(52.61±0.64)^{a}	(43.33±0.52)^{a}
	CR-2	(62.61±0.74)^{a}	(58.77±0.41)^{a}	(50.32±0.59)^{a}	(44.61±0.48)^{a}
	CR-3	(64.20±0.29)^{a}	(57.14±0.35)^{a}	(51.08±0.43)^{a}	(42.84±0.62)^{a}
	CR-4	(77.37±0.31)^{b}	74.28±0.68^{b}	(65.22±0.62)^{b}	(55.59±0.50)^{b}
	CR-5	(76.89±0.77)^{b}	(73.63±0.39)^{b}	(60.39±0.57)^{b}	(51.14±0.28)^{b}
	CR-6	(76.68±0.68)^{b}	(73.22±0.40)^{b}	(64.97±0.36)^{b}	(54.48±0.43)^{b}
	CR-7	(73.04±0.42)^{b}	(68.13±0.36)^{b}	(53.52±0.42)^{a}	(47.07±0.67)^{a}
	CR-8	(63.25±0.50)^{a}	(57.69±0.47)^{a}	(50.94±0.38)^{a}	(44.67±0.55)^{a}
Acid value/(mg KOH/kg)	CR-1	(3.68±0.21)^{a}	(7.07±0.34)^{a}	(13.41±0.42)^{a}	(24.22±0.33)^{a}
	CR-2	(4.23±0.17)^{a}	(6.83±0.26)^{a}	(14.29±0.33)^{a}	(26.50±0.28)^{a}
	CR-3	(3.94±0.25)^{a}	(7.22±0.20)^{a}	(12.34±0.24)^{a}	(25.67±0.45)^{a}
	CR-4	(2.11±0.16)^{b}	(4.37±0.31)^{b}	(7.92±0.25)^{b}	(17.08±0.39)^{b}
	CR-5	(2.52±0.32)^{b}	(4.91±0.15)^{b}	(9.34±0.27)^{b}	(20.54±0.44)^{a}
	CR-6	(2.45±0.19)^{b}	(5.38±0.28)^{b}	(10.69±0.40)^{ab}	(21.83±0.23)^{a}
	CR-7	(2.55±0.22)^{b}	(5.73±0.34)^{ab}	(12.38±0.24)^{a}	(24.21±0.31)^{a}
	CR-8	(3.72±0.25)^{a}	(7.46±0.21)^{a}	(13.40±0.28)^{a}	(25.38±0.23)^{a}
Peroxide value/(mmol O2-kg)	CR-1	(0.28±0.04)^{a}	(0.65±0.02)^{a}	(0.87±0.04)^{a}	(1.58±0.07)^{a}
	CR-2	(0.25±0.03)^{a}	(0.59±0.03)^{a}	(0.91±0.06)^{a}	(1.34±0.08)^{a}
	CR-3	(0.34±0.03)^{a}	(0.61±0.02)^{a}	(0.94±0.03)^{a}	(1.49±0.05)^{a}
	CR-4	(0.25±0.01)^{a}	(0.31±0.01)^{b}	(0.63±0.07)^{b}	(1.15±0.04)^{b}

CR-5	(0.26±0.03)^{a}	(0.42±0.01)^{b}	(0.74±0.02)^{b}	(1.26±0.06)^{ab}
CR-6	(0.30±0.02)^{a}	(0.41±0.02)^{b}	(0.69±0.05)^{b}	(1.29±0.05)^{ab}
CR-7	(0.21±0.02)^{a}	(0.40±0.04)^{b}	(0.75±0.03)^{b}	(1.45±0.03)^{a}
CR-8	(0.27±0.03)^{a}	(0.58±0.03)^{a}	(0.92±0.08)^{a}	(1.32±0.04)^{a}

Results are expressed as mean value±standard deviation. Different letters in superscript indicate significant difference between the samples in the same row (corresponding to the same batch). Sample abbreviations are given in Table 1

At the beginning of the study, we found small peroxide values (less than 1.00 mmol O_2/kg) in our sausages. Lorenzo *et al*. (*42*) states that starter culture used in fermented sausages inhibit the formation of peroxide values during storage, while Falowo *et al.* (*43*) indicates that slower lipid spoilage in fermented sausages results from a decrease of moisture and the denaturation of enzymes in the meat during storage. In our study, the highest peroxide values was found in control samples of cold-smoked sausages, and the smallest in samples with taxifolin and starter culture. Thus, the changes in peroxide values in fermented sausage samples indicate that taxifolin effectively inhibits chain reactions occurring in lipid peroxidation processes. These results coincide with the findings of Anastasiya *et al.* (*44*), who stated that the taxifolin even at the minimal mass fraction of 0.001 % inhibits the oxidation of the lipid fraction in the minced meat, because it reduces peroxide values by 58.60 % compared to the minced meat produced by the traditional recipe. Bakalivanova *et al.* (*45*) also stated that taxifolin has a beneficial effect on lipid peroxidase and is suitable for use in sausage production as an antioxidant.

On the first day of the study, the smallest acid value was determined in recipes with CR-4, CR-5, CR-6 and CR-7 (2.11±0.16, 2.52±0.32, 2.45±0.19 and 2.55±0.22 mg KOH/kg, respectively) ($p<0.05$). Gonzales *et al.* (*16*) states that flavonoids are able to bind metals that are capable of catalysing many biological processes such as fat hydrolysis. These results are confirmed by Topal *et al.* (*2*) who also found that taxifolin can bind free radicals and metal ions. At the end of the study (181 days), the significantly lower acid value was determined only in the sample with CR-4 ((17.08±0.39) mg KOH/kg) ($p<0.05$). In the samples, a strong positive correlation ($R=0.729$, $p<0.05$) and a negative correlation with pH was found between peroxide values and AV ($R=-0.831$ and $R=-0.874$, $p<0.05$ in both cases). We also found that acid value and peroxide values were influenced by the duration of storage ($p<0.05$).

Evaluation of colour properties of cold-smoked pork sausages

We determined that the colour parameters (*L*, a*, b**) of preparations and final meat products were significantly ($p<0.05$) influenced by the used taxifolin and starter cultures and storage time (Table 5). Meanwhile, some reports indicate that changes in colour of fermented sausage are influenced only by maturation time, but not by starter cultures (*46*). The redness (*a**) of investigated cold smoked sausages during storage decreased in all samples, but significantly decreased in samples CR-5, CR-6 and CR-7 alone from 33rd day of investigation ($p<0.05$).

Table 5. Effect of taxifolin with commercial recipes on colour during storage of cold-smoked pork

Colour	Samples	*t*(storage)/day			
		1	33	128	181
*L**	CR-1	(36.57±0.27)^{b}	(43.40±0.41)^{a}	(39.90±0.03)^{a}	(39.62±0.07)^{a}
	CR-2	(40.09±0.67)^{a}	(45.77±0.02)^{b}	(45.11±0.92)^{b}	(39.64±0.08)^{a}
	CR-3	(35.28±0.29)^{b}	(43.10±0.52)^{a}	(41.05±0.08)^{a}	(40.11±0.12)^{a}
	CR-4	(38.91±0.54)^{a}	(44.44±0.34)^{a}	(43.01±2.73)^{a}	(38.45±0.18)^{a}
	CR-5	(39.87±0.37)^{a}	(47.60±0.13)^{b}	(45.58±0.28)^{b}	(46.71±0.01)^{b}
	CR-6	(40.39±0.68)^{a}	(48.06±0.12)^{b}	(46.80±0.03)^{b}	(43.24±0.28)^{b}
	CR-7	(32.18±0.40)^{b}	(41.17±0.37)^{a}	(41.05±0.26)^{a}	(39.31±0.11)^{a}
	CR-8	(33.77±0.64)^{b}	(40.64±0.13)^{a}	(40.03±0.07)^{a}	(38.38±0.09)^{a}
*a**	CR-1	(15.69±0.49)^{a}	(25.89±0.36)^{b}	(16.36±0.03)^{b}	(14.80±0.04)^{a}
	CR-2	(16.14±0.38)^{ab}	(23.29±0.27)^{b}	(15.66±0.64)^{b}	(15.47±0.12)^{a}
	CR-3	(15.63±0.63)^{a}	(17.95±0.53)^{a}	(13.30±0.03)^{a}	(11.75±0.04)^{ab}
	CR-4	(15.99±0.31)^{a}	(20.99±0.17)^{b}	(14.14±2.62)^{a}	(15.79±0.02)^{a}
	CR-5	(18.34±0.49)^{b}	(20.45±0.31)^{b}	(15.87±0.11)^{b}	(14.15±0.03)^{a}
	CR-6	(17.52±0.61)^{ab}	(20.55±0.57)^{b}	(14.91±0.03)^{a}	(14.65±0.07)^{a}
	CR-7	(15.44±0.68)^{a}	(20.44±0.10)^{b}	(13.71±0.05)^{a}	(10.85±0.08)^{ab}
	CR-8	(14.42±0.72)^{a}	(14.40±0.14)^{a}	(12.84±0.02)^{a}	(8.37±0.05)^{b}
*b**	CR-1	(13.01±0.16)^{a}	(19.53±0.42)^{a}	(8.88±0.02)^{a}	(7.39±0.03)^{a}
	CR-2	(13.01±0.24)^{a}	(20.91±0.18)^{a}	(10.01±0.36)^{a}	(9.13±0.06)^{a}
	CR-3	(11.71±0.60)^{a}	(13.01±0.33)^{a}	(7.37±0.02)^{a}	(6.23±0.05)^{a}
	CR-4	(12.54±0.38)^{a}	(15.74±0.22)^{a}	(10.57±1.31)^{a}	(8.69±0.04)^{a}
	CR-5	(13.05±0.61)^{a}	(16.52±0.03)^{a}	(9.30±0.02)^{a}	(9.15±0.01)^{a}
	CR-6	(13.18±0.51)^{a}	(15.66±0.46)^{a}	(8.48±0.02)^{a}	(8.19±0.01)^{a}
	CR-7	(10.88±0.17)^{a}	(19.89±0.31)^{a}	(10.64±0.05)^{a}	(7.08±0.01)^{a}
	CR-8	(11.86±0.29)^{a}	(18.04±0.08)^{a}	(11.27±0.04)^{a}	(10.05±0.08)^{a}

Results are expressed as mean value±standard deviation. Different letters in superscript indicate significant difference between the samples in the same row (corresponding to the same batch). Sample abbreviations are given in Table 1

CONCLUSION

In this study, we evaluated the effect of taxifolin on the microbiological profile of cold-smoked sausages, as well as their acidity, biogenic amine content, and colour properties. The stability and antioxidative activity of taxifolin was also evaluated. It was found that taxifolin had an effect of increasing acidity in cold-smoked sausages during storage as shown by the results obtained, where lower pH values were found in samples CR-3 and CR-6. Taxifolin had an inhibitory effect on mould and yeast, and inhibited fat peroxidation processes, leading to lower acid values. A negative correlation between pH and acid value has also been found. Taxifolin and a commercial recipe with P. pentosaceus and S. xylosus could reduce the accumulation of total BA values, and also stabilize and slow down the rate of lipolysis and effectively inhibit the processes of lipid peroxidation as well.

On the other hand, taxifolin causes the color to fade, which could be unfavorable effect, and modification of sausage fermentation would be needed.

Taxifolin should be studied in detail to improve the food processes and provide maximum beneficial health effects to the consumers with optimum nutritional and functional properties. It is very important to continue working with different meat products and taxifolin to better understand the effect of interactions of different compounds. The outcome of this study can help develop new fermented meat products with beneficial health aspects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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