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Effect of taxifolin on physicochemical and microbiological parameters of dry-cured pork sausage

Anita Rokaityte[1], Gintare Zaborskiene[1,2], Sonata Gustiene[1], Raimondas Raudonis[3], Valdimaras Janulis[3], Galina Garmiene[2], Arturas Stimbirys[1]*

[1]*Department of Food Safety and Quality, Veterinary Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania*

[2]*Food Institute, Faculty of Chemical Technology, Kaunas University of Technology, Kaunas, Lithuania*

[3]*Department of Pharmacognosy, Faculty of Pharmacy, Lithuanian University of Health Sciences, Kaunas, Lithuania*

**Corresponding author: anita.rokaityte@lsmuni.lt*

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Abstract: The effect of taxifolin (TXF) with starter cultures (SC), such as *Leuconostoc carnosum*, or a mixture of strains *Pediococcus pentosaceus* and *Staphylococcus xylosus,* on the TXF stability was evaluated. *UPLC* analysis demonstrated that after 181 days of storage total TXF content was the highest in samples with TXF and *L. carnosum* (60%), compared to the 1st day of storage. The sausages with TXF and the mixture of *P. pentosaceus* and *S. xylosus* (56%) followed next. The samples treated only with TXF retained 40% of TXF, compared to the 1st day of storage. TXF had no significant effect on the growth of lactic acid bacteria. The accumulation of biogenic amines (BA), including histamine and putrescine, was more effectively reduced in sausages inoculated with the TXF plus *P. pentosaceus* and *S. xylosus* mixture. Using this mixture, the rate of lipolysis and processes of lipid oxidation were effectively slowed down. Fatty acid (FA) composition was stable in all cases.

Keywords: biogenic amines; fatty acid; *L. carnosum*; *P. pentosaceus*; *S. xylosus*

The application of bioflavonoids as natural antioxidants for treatment of dry-cured pork sausages with SC has not been discussed in the available literature. The taxifolin (TXF) (also known as dihydroquercetin) is a member of the group of flavanones (Vladimirov *et al.* 2009). The satisfactorily pure TXF may be extracted from Siberian larch (*Larix sibirica Ledeb.*). TXF has a positive effect on human health, as a typical flavonoid inhibits free radical formation (Trouillas *et al.* 2004), influences the physical properties of lipids in biological membranes (Theriault *et al.* 2000), ameliorates cerebral ischemia-reperfusion injury (Wang *et al.* 2006) and activates the formation of collagen fibres (Tarahovsky *et al.* 2007). TXF has many health-promoting effects, but is unstable to the light, pH, and thermal treatment (West & Mauer 2011; Kumar *et al*. 2016; Bobolaki *et al*. 2018). Besides, little is known about the degradation behaviour of TXF throughout the storage time. Knowledge about the stability of TXF in food processing is important in order to predict the duration of physiological effects in food and beverages (Shiko *et al*. 2009). The food additives authorized for use in Europe are reported in European Regulation (EC) No. 1129/2011, as well as the levels of their maximum permitted usage in the food where their use is permitted. TXF is not reported in

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the Annex II to the aforementioned Regulation, and it is therefore not to be considered as an authorized food additive in Europe.

In the future, TXF could be used as a natural antioxidant and antimicrobial additive in the food industry (Wang *et al.* 2011; Topal *et al.* 2016), such as dry fermented sausages with SC. The addition of selected SC 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 product (Essid *et al.* 2013, Ciuciu Simion *et al.* 2014).

The aim of this work was to evaluate the effect of mixtures of TXF combined with different SC on the physicochemical and microbiological parameters in order to select the most suitable mixture for the safety and quality of dry fermented pork sausages during storage.

MATERIAL AND METHODS

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

Sausage production and sampling procedures. Six different batches of pork sausage were manufactured according to traditional techniques (60 sausages in separate batches): two batches – with addition of different SC (Chr. Hansen, Denmark) in a proportion defined by the manufacturer in each case, the other two batches – with addition of SC with TXF, one batch – only with TXF and one control batch. The batches were named as follows: B-SF-43 (*L. carnosum*); T-SPX (*P. pentosaceus* and *S. xylosus*); B-SF-43 (*L. carnosum*) plus TXF; T-SPX (*P. pentosaceus* and *S. xylosus*) plus TXF; TXF only with TXF; Co-batch – control without SC and TXF. Pork sausages formulation includes whole pork muscle and back fat cuttings (80%), raw pork ham (20%), NaCl (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), sodium nitrite (0.15 g/kg) and potassium nitrate (0.15 g/kg). The whole muscle cuttings and raw ham were minced through a 13 mm diameter mincing plate and vacuum mixed together with the other ingredients for 3 minutes. The mix was maintained at 4°C for 24 h and then stuffed into natural casings with a diameter of 30 mm and a length of 5 cm. The sausages were fermented for 2 days at 21°C and 85% of relative humidity (RH) and then transferred into a drying ripening chamber where they were kept for 18 days at 25–16°C and 93–82% RH. Made dry-cured sausages were kept for 181 days at 15°C and 75% RH. Sausages after 1, 33, 128, and 181 days of storage were taken for TXF stability, physicochemical parameters and microbiology analysis. The analyses were carried out in triplicate and experiments were repeated three separate times.

Determination of TXF in dry-cured sausages using UPLC. All the reagents and standards were of analytical grade. HPLC-grade acetonitrile, trifluoroacetic acid (TFA), TXF from Sigma-Aldrich (Switzerland). Deionized water was acquired from a Milli-Q purification system (Bedford, USA).

Sausage samples (1 g) were weighed and transferred into a 100 ml stoppered conical flask. EtOH (96% 100 ml) was added, and placed on a rotary shaker (MaxQ 4000; Thermo Scientific, USA) under agitation (300 rpm) for 30 minutes. The mixture was filtered through filter paper 601A (Whatman, UK). The clear portion kept in a freezer for at least 20 min, for the *separate* extraction of *fat.* The mixture was next centrifuged at 8500 rpm for 20 min at 4°C. The upper phase was filtered through a 0.2 µm pore-size syringe filter (Acrodisc LC13 PVDF; Sigma-Aldrich, Canada) and injected into the HPLC unit. Dry matter content was determined by drying a homogenized sample in an oven at 105°C until no difference in weight was observed.

Chromatographic analysis was carried out with a Waters Aquity UPLC system consisting of binary solvent manager, auto sampler, column manager and PDA detector (Waters, USA). The UPLC column was a 2.1 × 100.0 mm Acquits UPLC C18 BEH (Waters) containing 1.7 µm particles. The mobile phase consisted of 0.1% TFA 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 in 3 min to 10%, and was held at 10% for 1 minute. 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).

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Moisture content of the dry-cured sausages samples was obtained according to ISO 1442:2000.

pH measurement. The pH of sausages was measured according to the standard method for determination of meat pH (EN ISO 2917:2002). pH measurements were carried out using a PP-15 pH-meter (Sartorius, Germany).

FA content. The amount of FA was determined by the method of gas chromatography using flame ionization detector. For the analysis of FA, the samples were prepared according to the standard EN ISO 12966-2:2011. FA was methylated using anhydrous KOH methanol solution. Chromatographic analysis of FA methyl esters was performed using gas chromatograph Shimadzu GC – 17A, using BPX – 70, 120 m column following the methodology determined in EN ISO 15304:2003/AC:2005 2. The FA methyl esters (FAME) were identified by comparison of each retention time with Supelco 37 Component FAME mix 47885-U (Sulpeco, Germany).

Determination of DPPH free radical scavenging activity. The method used by Takao *et al.* (1994) was adopted with suitable modifications from Kumarasamy *et al.* (2007).

Acid value (AV). AV of the extracted lipids was determined according to EN ISO 660:2009-10 procedure (Wang *et al.* 2010).

Peroxide value (POV). POV of the studied lipids is determined by standard (EN ISO 3960:2010) iodometric method and was presented as meqv O_2/kg lipids (AOAC 2012).

Microbial analysis. Samples of 10 gwere taken at random for each sample and aseptically weighed into a sterile stomacher bag with 90 ml of sterile Buffered peptone water 0.1% (w/v) (REF 611014; Liofilchem, Italy) and homogenized for 1 min in a model 400 Stomacher (Seward Medical, UK). Serial decimal dilutions were made and lactic acid bacteria (LAB) were determined by plate count on MRS Agar CM 0361 (Oxoid, UK) after an incubation at 30°C for 120 h; total *Enterobacteriaceae* were determined on Violet Red Bile Glucose Agar (Merck, Germany) after an incubation at 37°C for 24 h; presumptive *Pseudomonas* spp. were determined on the Pseudomonas Agar Base CM 0559 (Oxoid, UK) supplemented with CFC (Oxoid, SR 0103, UK) after aerobic incubation of the dishes at 25°C for 48 hours. After incubation, plates with 10–300 colonies were counted according to ISO 7218/2007. The microbiological data were transformed into logarithms of the number of colony forming units (CFU/g).

Biogenic amines (BA) content. A reversed-phase high-performance liquid chromatography (RP-HPLC) method was used for the quantitative analysis of the BA – tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine. The whole cured sausage (edible parts) was cut into small pieces and mashed mechanically using a homogeniser (Moulinex). BA was extracted from a homogenized sample with 0.4 mol/l perchloric acid. The derivatization of samples was carried out using the modificated methodology of Ben-Gigirey *et al.* (2000). Extract was derivatised for 45 min by dansyl chloride (5-dimethylaminonaphtalene-1-sulfonylchloride) solution in acetone at 40°C. The samples were filtered through 0.45 µm membrane filter (Millipore Co., USA), 10 µl was injected into chromatographic system 1200 Series (Agilent, Germany). Analysis was performed using LiChro column CART® 95 125-4.

Statistical analysis of the data. Data were statistically analysed using the SPSS 20.0 software (SPSS Inc., USA). Data are the means of experiments performed in *triplicate.* Differences between dates were evaluated by the analysis of variance method (one-way ANOVA) with a significant level of $P < 0.05$ (Draper *et al.* 1998). Multiple comparisons were estimated by Fishers Least Significant Difference method and 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 $P < 0.05$ or $P < 0.01$.

RESULTS AND DISCUSSION

TXF stability. A far more objective view of changes in TXF contents in the dry-cured sausages can be obtained after recalculation of the TXF content on a dry weight basis. This recalculation eliminates the effect of variable water content on changes in TXF content during production. About 28% was weight loss in all sausages during ripening and storage but there was no significant difference between the batches. After 181 days of storage total TXF content, compared to the 1st day of storage, was the highest in samples with B-SF-43+TXF (60%), followed by the sausages from T-SPX+TXF (56%) (Figure 1). While, the samples treated only with TXF retained 40% of TXF, compared to the 1st day of storage. UPLC analysis demonstrated that TXF was more stable in samples with SC. It could be related to a stronger acidification during the first 33 days. These findings are in concordance with those reported by Zhang

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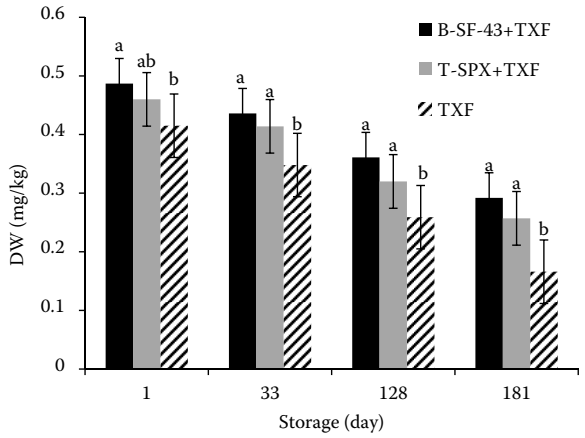

Figure 1. Content of TXF during storage of dry-cured sausages on a dry weight basis

[a,b]significant to the same days of storage

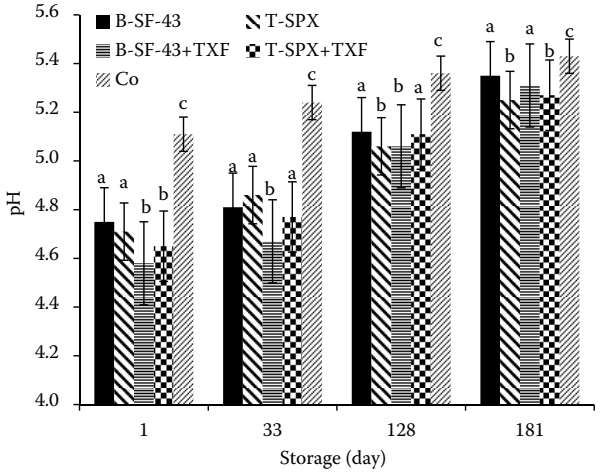

Figure 2. Effect of TXF with SC on the evolution of the pH value during storage of dry-cured sausages

[a,b,c]significant to the same days of storage

et al. (2013) who noticed that flavanol astilbin in alkaline solution was decreased over storage time, and the higher the pH value, the faster the degradation.

pH values. Values corresponding to pH values throughout ripening are summarized in Figure 2. The inoculation of the SC resulted in a stronger acidification during the first 33 days of production. The decrease of the active acidity pH is due to the carbohydrate fermentation processes, which accumulate organic acids, mainly lactic acid, during the storage period (Naila *et al.* 2010). At the end of the study, the final pH value of dry-cured sausages was between 5 and 6. This data coincided with Berardo *et al.* (2017), who claimed that using the "Nordic" sausage technology, the final pH value is 5 and less, while using the "Mediterranean" technology, the final pH value is between 5 and 6.

FA composition. Regarding to individual FA, palmitic (C16:0) presented the highest values, followed by oleic acid (C18:1), linoleic (C18:2n6) and stearic (C18:0) acids. The sum of these four FA represented between 88 and 90% of total FA. Statistical analysis displayed that FA profile was not affected by TXF and SC, considering that the major release of the latter was probably due to endogenous lipases. These findings are in concordance with the findings reported by Essid and Hassouna (2013), and Lorenzo *et al.* (2014) who noticed that inoculated and control sausages (co) displayed the same FA composition.

Antioxidant activities. In the present investigation, the DPPH assay was used to evaluate the radical-scavenging activity of TXF with and without SC in sausages. The antioxidant capacity of TXF was lower than the values of the TXF with SC (Figure 3). This indicates a stronger antioxidant activity of TXF in relation to the SC, traditionally used in the fermented production.

The high levels of AV (higher than 2.00 mg KOH/g lipids) were found in all examined samples on the first day of the experiment (Figure 4). These results indicate that the hydrolytic changes of the lipids began during the sausage ripening and drying process. The changes determined in the AV of the samples showed that the addition of TXF with SC slows down the rate of lipolysis. Gonzales *et al.* (2015) states that flavonoids are able to bind metals those are capable of catalysing many biological processes, including fat hydrolysis. These results are confirmed by Topal *et al.* (2016) who also found that TXL can bind free radicals and metal ions.

Up to 33 days of experiment in all analysed samples, a small quantity (less than 1.00 meqv O_2/kg) of lipid hydroperoxides was determined (Figure 5). Lorenzo *et al.* (2017) states that SC used in fermented sausages inhibit the formation of POV during storage. While Falowo *et al.* (2014) indicates that slower lipid spoilage in fermented sausages results from a decrease of moisture and the denaturation of enzymes in the meat during storage. The determined changes of the POV in samples show that the addition of TXF effectively inhibits the chain reactions generating during the processes of lipid peroxidation. On 181th day of experiment the highest POV levels were registered in the control samples (3.22 ± 0.21 meqv O_2/kg) and the smallest in samples with TXF and SC (B-SF-43+TXF and T-SPX+TXF, respectively 57%

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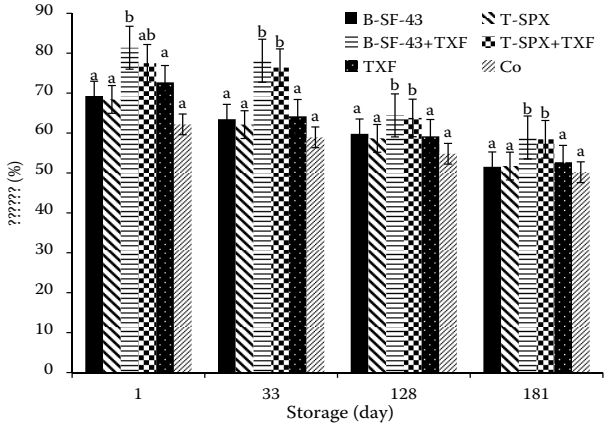

Figure 3. Effect of TXF with SC on DPPH radical scavenging activity during storage of dry-cured sausages

[a,b]significant to the same days of storage

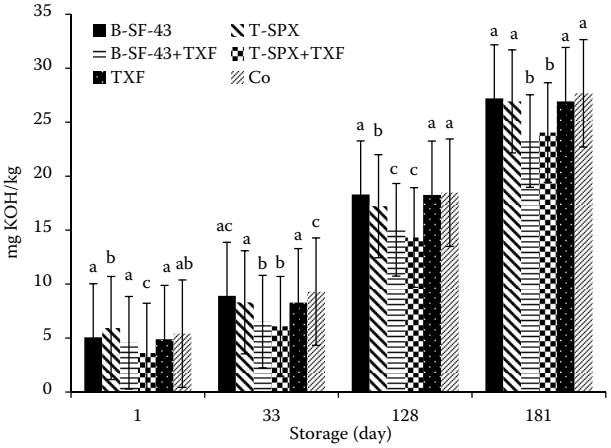

Figure 4. Effect of TXF with SC on Acid value during storage of dry-cured sausages

[a,b,c]significant to the same days of storage

and 36% less comparing with control).This indicates that TXF effectively inhibits chain reactions occurring in lipid peroxidation processes. These results coincide with Anastasiya *et al.* (2007), which stated that the TXF even at the minimal concentration of 0.001% inhibits the oxidation of the lipid fraction in the minced meat, because it reduces POV by 58.6% compared to the minced meat produced by the traditional recipe. Bakalivanova *et al.* (2012), also stated that TXF has a beneficial effect on lipid peroxidase and is suitable for use in sausage production as an antioxidant. Scientific literature indicates that the most suitable concentration of TXF for meat products, depending on the fat content, is from 0.006% to 0.04% Gonzales *et al.* (2015).

Microbial environment. Initial LAB counts in inoculated batches with SC were three log units higher than those in the Co and only with TXF batches (7.97, 8.41, 8.63, 8.50, 5.61, and 5.68 log CFU/g for B-SF-43, T-SPX, B-SF-43+TXF, T-SPX+TXF, TXF, and Co-batches, respectively) on the 1st day of experiment (Figure 6). The maximum level was observed on the 1st day of experiment and then a slight decrease was observed to reach at the end of storage. Our findings are similar to those reported by Kameník *et al.* (2013), who observed that the reproduction of LAB occurs during the first days of fermentation and the population density peaks at 10^9 cells/g.

During storage of inoculated sausages, *Enterobacteriaceae* displayed a strong decrease and reached values

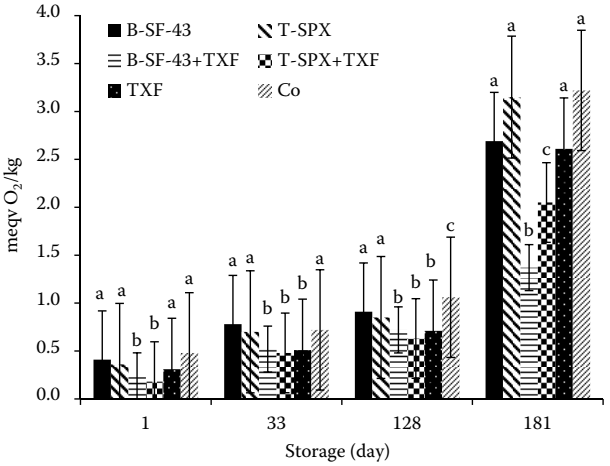

Figure 5. Effect of TXF with SC on Peroxide value during storage of dry-cured sausages

[a,b,c]significant to the same days of storage

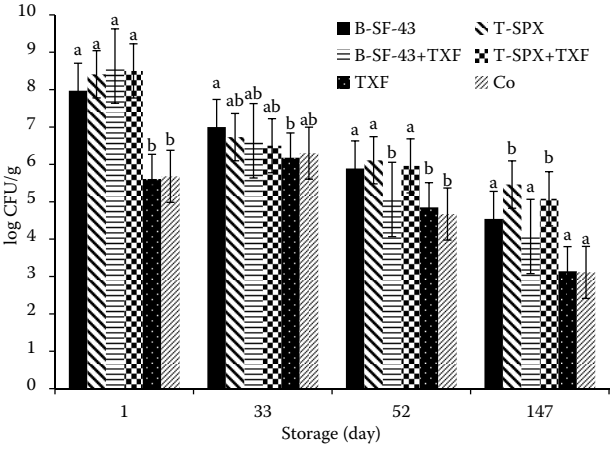

Figure 6. Effect of TXF with SC on the evolution of the counts of LAB during storage of dry-cured sausages

[a,b]significant to the same days of storage

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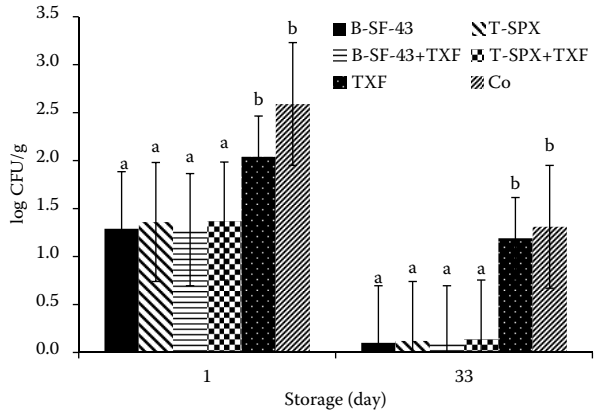

Figure 7. Effect of TXF with SC on the evolution of the counts of Enterobacteriaceae during storage of dry-cured sausages
[a,b]significant to the same days of storage

below 1 log CFU/g, whereas, in the sausages only with TXF and Co, these groups presented a slight decrease and reached the level of 1.19 and 1.31 log CFU/g, respectively on the 33rd days of the experiment ($P < 0.05$) (Figure 7). *Pseudomonas* spp. displayed a strong decrease and reached values below 1 log CFU/g, whereas, in the sausages without SC groups presented a slight decrease and reached the level of 1.03 and 1.23 log CFU/g, (respectively, TXF and control sausages) on the 33rd day of the experiment ($P < 0.05$) (Figure 8). This decrease for the *Enterobacteriaceae* and *Pseudomonas* spp., is partially explained by the pH decrease (respectively, $r = 0.575$ and 0.605, when $P < 0.01$ in all cases). These results coincide with Lorenzo *et al.* (2012) and Ciuciu Simion *et al.* (2014), which indicates that the decrease in the number of spoilage bacteria in sausages is most often due to a decrease in the pH value due to the used SC.

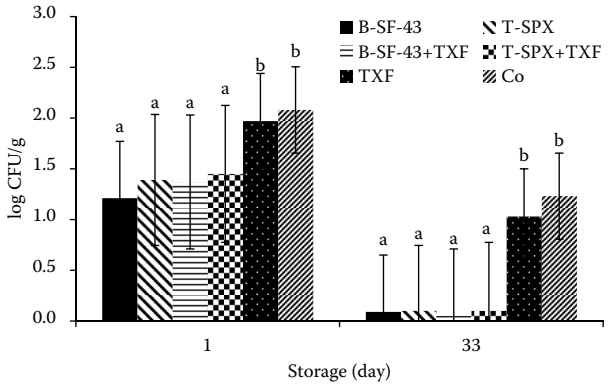

Figure 8. Effect of TXF with SC on the evolution of the counts of *Pseudomonas* spp. during storage of dry-cured sausages
[a,b]significant to the same days of storage

From the results of the current work it seems that the inclusion of the SC substantially contributes to the decrease of the Gram-negative bacteria throughout the ripening, While only TXF had no antibacterial effect.

BA accumulation. BA can be detected in raw materials and food products that are formed during metabolic processes. The main BA produced in the sausage during fermentation are putrescin, cadaverine and tyramine (Ojha *et al.* 2015). In our study, mainly contain were putrescine (between 33.91 and 107.96 mg/kg), tyramine (between 15.29 and 39.21 mg/kg), cadaverine (between 17.01 and 32.06 mg/kg) and spermine (between 2.57 and 10.79 mg/kg) at the end of experiment. After 33 days of experiment, T-SPX+TXF group presented significantly ($P < 0.05$) lower histamine values (2.45 mg/kg) comparing with all sausages groups (between 7.13 and 17.11 mg/kg) and putrescine (17.23 mg/kg) comparing with all sausages groups (between 39.50 and 58.72 mg/kg). A significant ($P < 0.05$) higher amount of tyramine was observed in T-SPX batch compared with T-SPX+TXF (respectively, 39.21 ± 2.57 and 24.91 ± 2.61 mg/kg) in the end of the experiment. The reduction in tyramine formation through natural antioxidant extracts is important with respect to human health because tyramine causes migraine headaches, increased blood pressure and an increase in noradrenalin as has been previously reported by Ruiz-Capillas and Jiménez-Colmenero (2004). The total BA content in sausages inoculated with the T-SPX+TXF mixture was 21 and 43% lower than that in T-SPX and control sausages ($P < 0.05$) in the end of the experiment. Besides in sausages inoculated with the B-SF-43+TXF mixture was 29% lower than that in B-SF-43 batch, wherein the presented highest total BA content (188.29 ± 4.09 mg/kg). According to Nout (1994), a level of 1.000 mg/kg of total BA in sausage products elicits toxicity in humans. In this study, the total BA contents of sausages were lower than recommended hazardous levels, indicating good manufacturing practice for pork sausage. The accumulation of BAs can more effectively inhibit the mixed cultures with TXF.

CONCLUSIONS

TXF conditions 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 food

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sources and TXF to better understand the exact effect of interactions of different compounds. The outcome of this study can help develop new meat products with better microbiological parameters and beneficial health aspects because mixed TXF with cultures can stabilize and slow down the rate of lipolysis and effectively inhibit the processes of lipid peroxidation. Besides this mixtures can reduce the accumulation of total BA values including histamine and putrescine.

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