Article

Reducing N-nitrosamine content in grilled pork steaks marinated with selected strains of lactic

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Abstract: The results show that seven types of N-nitrosamines: N-nitrosodiethylamine (NDEA), N-Nitrosopiperidine (NPIP), N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR), N-nitrosodipropylamine (NDPA), N-nitrosodibutylamine (NDBA) and N-nitrosodiphenylamine (NDphA) were reduced after heat treatment of pork steaks marinated with *L. plantarum* MI-LP1, S. carnosus LM-SC1, *S. vitulinus* LM-SV1. Selected LAB suppressed the formation of putrescine and spermine, lowered acid and peroxide values, which were significantly lower than in the control samples, then P≤0.05 in all cases. The nitrites and nitrates amounts were significantly decreased in pork steaks samples marinated with *L. plantarum* MI-LP1 and *S. carnosus* MI-SV1 strains. These results presented that selected lactic acid bacteria could be used for marinating pork steaks to improve quality characteristics and reduce N-nitrosamine formation during grilling.

Keywords: N-nitrosamine; bio preservatives; nitrate; nitrite

1. Introduction

United Kingdom dietary guidelines, World Cancer Research Fund/American Institute for Cancer Research recommend limiting processed meat intake, because higher intakes of red processed meat were associated with a higher risk of colorectal cancer [1,2]. Sodium nitrite can delay lipid oxidation, give the product a typical cured meat flavor, promote the color formation and inhibit pathogenic bacteria, hence they are considered indispensable in many types of meat [3]. However the formation of carcinogenic N-nitrosamines is based on the reaction of sodium nitrite and secondary amines, which give rise to mutagenic, teratogenic or carcinogenic consequences in the human body [4]. N-nitrosamine in foods may be risk factor for human cancers, for example, cancers of the esophagus, nasopharynx ,and stomach [5–7]. Some plants used in meat processing contain nitrates and nitrites whose amount can be potentially high, for example: in oregano nitrate varied from 106.06 mg/kg to 415.58 mg/kg [8], in mustard nitrite – 12.07 mg/kg, nitrate – 15,48 mg/kg [9]. Therefore, nitrite in processed meat products should be limited as much as possible.

Recent research has revealed that microbial fermentation may be a suitable method to reduce N-nitrosamine formation [10–12]. According to Paik and Lee [13], *Lactobacillus brevis* KGR3111, *Lactobacillus sakei* KGR 4108 and Lactobacillus plantarum KGR 5105 isolated from kimchi is acceptable way to reduce nitrite content. LAB, isolated from traditional Dong pork products (Nanx Wudl) was used in the production of Chinese fermented dry sausages, the nitrite content of the inoculated sausages rapidly declined during ripening, according to Chen et al [14].

The most common N-nitroso-compounds in processed meat are NDMA, NDEA, NDPA, NDBA, NDphA, NPYR, NPIP, and Nitrosomorpholine (NMor) [15,16]. Factors

that can influence the formation of N-nitrosamines in meat products is meat type, nitrite, biogenic amines content, amino acid decarboxylase activity of microorganisms, moisture, temperature, pH, and potential redox. Physical or chemical means to reduce nitrosamine content are abundant and research concentrates on them, but the solution may be the increasingly observed connection between LAB strains and nitrosamine content.

Our research work investigated replacing chemical additives (e.g. sodium nitrite) in meat processing with more sustainable and safer biopreservatives, i.e., herbs and lactic acid bacteria (LAB) cultures isolated from natural sources. In all likelihood, it could help to reduce carcinogenic N-nitrosamines in grilled meat steaks. Therefore the aim of our studies was to investigate the formation of N-nitrosamine in grilled pork steaks marinated with selected strains of *Lactobacillus spp*. and *Staphylococcus spp*. bacteria's. Those studies investigate the effect of biofermentation by LAB on the safety of marinated steaks with special emphasis on amounts of nitrites, nitrates, biogenic amines, D lactic acid, and peroxides.

2. Materials and Methods

2.1. Selection of LAB

Two LAB strains from the Food Institute's (Kaunas University of Technology) collection (*Lactobacillus plantarum* MI-LP1 and *Lactococcus lactis subsp*. *Lactis biovar diacetylactis* MILLd57) were selected for this study. These strains had the best antimicrobial properties. Strains were stored at -72 °C in "VIABANK" system (*Medical Wire and Equipment*, UK). LAB strains were revived for 24 h in the modified MRS broth (Biolife, Italy) at 30 °C. Seventeen cultures were isolated from the cold smoked meat products and identified using Maldi Biotyper MSP Identification Standard method. Isolates were assigned to 3 species *Staphylococcus carnosus*, *Staphylococcus vitulinus*, and *Enterococcus faecalis*. *Staphylococcus carnosus* LM-SC1, *Staphylococcus vitulinus* LM-SV1, and *Enterococcus faecalis* MI-EF1 were included into Institute's collection and 2 of them were used for experiments. All bacterial cultures were inoculated to each group of samples at level 106 CFU/g.

2.2. Preparation of the LAB cultures for the experiment

L. plantarum MI-LP1 and *Lactococcus lactis subsp*. lactis biovar *diacetylactis* MI- LLd57 cultures were prepared for experiments: modified MRS broth was inoculated with 1 % of the fresh culture (at 18 h-72 h age) in the same medium and incubated for 72 h at 30°C.

Then the culture was centrifugated at 4000 rpm 3 times for 30 min in a sterile 0.9% NaCl solution. The precipitate was washed with a sterile physiological solution (in a ratio 1:20) to receive pure cell mass and that mixture was centrifugated again. The number of *L. plantarum* MI-LP1 in the precipitate was approximately 1.5·109 CFU/g.

2.3. Meat samples and treatment

Three choose randomly whole pork loins (*Longissimus lumborum*) were taken from a local slaughterhouse (Kedainiai, Lithuania) at 72 h. *post-mortem* and were used for the studies. The average weight of slaughtered pigs were approximately 90 kg. The pork loins were considered as a relatively homogenous muscle with an average pH value of 5.6±0.1. Using low-density polyethylene bags, at a temperature of + 4°C, the pork loins with external fat were stored until the subsequent experiment. Pork loins were cut into a total of 50 chop samples of approximately the same weight and size (20 g and 2 cm × 2 cm × 2 cm) for marinating. The chop samples were randomly divided into the following five treatments for the experiments to obtain an even distribution.

The following five treatments were applied:

1. marinate with *Lactobacillus plantarum* (marinating with *L. plantarum* MI-LP1, at 4°C);

2. marinate with *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (marinating with *Lactococcus* lactis subsp. *Lactis* biovar *diacetylactis* MI-LLd57, at 4°C);

3. marinate with *Staphylococcus carnosus* (marinating with *Staphylococcus carnosus* LM-SC1, at 4°C);

4. marinate with *Staphylococcus vitulinus* (marinating with *Staphylococcus vitulinus* LM-SV1, at 4°C);

5. Control group - 1.5% acetic acid marinate (marinating using 9% food vinegar, at 4°C).

The basic composition of the marinate in all treatments was: white sugar 0.5 %, sodium chloride (NaCl) 1.0 %, mustard 0.5 %, coriander 0.5 %, oregano 0.30%, black peppers 0.30%. All ingredients were purchased from the company Sirmulis Lithuania. The meat samples were placed separated into five tanks and were left for marinating of 5 days period.

2.4. Physicochemical analysis of marinated pork steaks

The pH of the meat samples was measured according to the standard method EN ISO 2917:2002 [17]. Measurements were carried out using a PP-15 pH meter (Sartorius Professional meter for pH Measurement, Germany). The pH meter those calibrated at two points in pH 4.01 and 7.00 with standard buffers before analysis (Sigma Aldrich, Saint Louis, USA). Temperature corresponded to the approximate muscle temperature. The extraction of meat lipids was carried out according to AOCS (1997). Acid values of the meat lipids extracted lipids were determined according to EN ISO 660:2020 [18]. Peroxide values lipids were evaluated according to EN ISO 390:2017 [19].

Biogenic amines - putrescine, cadaverine, histamine, tyramine, and spermine were determined by the reversed-phase high-performance liquid chromatography (RP-HPLC) method. Samples were catted into small pieces and mechanically homogenized (Moulinex Masterchef 20, Nieune, France). Biogenic amines from the homogenized samples were extracted with 0.4 mol/L perchloric acids. The derivatization was performed according to the modified method of Ben-Gigirey et al. [20]. The extracts were derivatized with dansyl chloride (5-dimethylaminonaphtalene-1-sulfonylchloride) solution in acetone for 45 min. at 40 °C. Samples were filtered through a membrane filter (0.45 μm Millipore Co., Bedford, MA, JAV), 10 μL of each filtrate was injected into chromatographic column - LiChro column CART® 95 125-4 (Merck, Darmstadt, Germany). The analysis was performed using a chromatographic system (Aligent 1200 Series, Waldbronn, Germany).

The spectrophotometer (Minolta CM-2002; Osaka, Japan) was used for measuring of Meat sample's color. Parameters *L *, a **, and *b ** were measured in the reflection mode (Wang et al., 2015). Color Changes in the surface of meat samples during storage were monitored by recording the CIE L* (lightness), b* (yellowness), and a* (redness) values using a reflectance spectrophotometer (Minolta CM 2002, Osaka, Japan) using D65, 2° observer and 8 mm aperture diameter.

D, L-lactic acid levels were determined using a D, L-lactic acid analysis kit (KD-Late kit, Megazyme International Ltd., Co. Wicklow, Ireland). This is an absorbance-based spectrophotometer assay and was performed using and Helios Gamma spectrometer (Thermo Scientific, UK).

The official ISO 2918:1975 [21] method Meat and meat products — determination of nitrite content (Reference method) were used for the determination of nitrite content in meat samples.

The content of nitrate in examined meat products was determined according to the standard ISO 3091:1975 [22] Meat and meat products – determination of nitrate content (Reference method).

Standards of N-nitrosamines: NDMA, NDEA, NPYR, NDPA, NPIP, NDBA, and NDpheA were acquired from Sigma-Aldrich GmbH, Merck, Switzerland. Standard solutions for the calibration curve were prepared to dilute with methanol (Sigma-Aldrich, Saint Louis, USA) at concentrations of 0.01, 0.05, 0.1, 0.2, and 1 μg /ml, and kept at 4°C. Water was purified using the „Elix- Milli-Q" system (Millipore, Bedford, MA, USA). All grilled pork steaks samples were kept at -18 °C before nitrosamine determination.

Twenty grams of crushed samples were blended (Philips, Germany) and put into a 100 ml glass. Forty milliliters of dichloromethane (Sigma-Aldrich GmbH, Merck,

Switzerland) were also added. The samples were there mixed for 30 minutes in an ultrasonic bath (USC 300 TH, VWR International Ltd, UK) at 500 W.

The extraction procedure was repeated with 40 ml dichloromethane to cafully evaporate volatile nitrosamines. Then, the liquid extract was mixed and filtered through sodium sulfate (Sigma-Aldrich, Merck, Oakville, ON, Canada). Using a rotary evaporator at 30 °C, the sample was vaporized until the liquid concentrated to about 1 ml. One ml of methanol was added to a test tube and mixed for 1 minute (Vortex 4 digital, IKA® Werke GmbH & Co, Staufen, Germany). Finally, the sample solution was filtered through 0.45μm syringe filter (Millipore Co., Bedford, MA, USA) for GC-MS analysis. For the evaluation of the amount of N-nitrosamine gas chromatography with mass spectrometry was used (PerkinElmer Clarus 680, Linealibera, USA). For the optimization of the technique HP-5MS capillary column was used (30mx0,25mmx0,25μm, PerkinElmer Elite TM 1, Linealibera, USA). The temperature of GC furnace was programmed like that: initial temperature - 40 °C (kept for 3 minutes), then increased to 240 °C in 15 °C / minute increments, keeping for 1 minute. Standards of N-nitrosamines were used for identification, according to outgoing time. Nitrite and nitrate content in the final product was determined according to the previously mentioned methodology.

2.5. Statistical analysis of the data using GLM - Univariate model

The measurements of the experiment were triplicate, and statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, Illinois, USA). Data were presented using descriptive statistics and normal distribution analysis methods. Identified outliers have been removed. Predicted means and standard errors were generated from the models. The influence of the tested parameters of marinated steaks: pH, acid, peroxide value, amounts of Lactic acid L, D isomers, and the content of biogenic amines, nitrite, and nitrate on the N-nitrosamine levels was determined by One-way Analysis of Variance (ANOVA). Multiple comparisons of group means were calculated using Tukey's test. The differences were considered as significant at $P<0.05$. Fixed effects for the N-nitrosamine model included acid, peroxide value, and amounts of lactic acid isomers- Non-significant terms were removed from models using the stepwise backward elimination method. In GLM - Univariate model the fixed factors were: treatment and storage day and the interaction of treatment x storage day; the random factor was replication.

3. Results

3.1. Acidity of marinated pork steaks

The pH is a relevant factor affecting the activity of amino acid decarboxylase, which is stronger in an acidic environment, with a pH between 4.0 and 5.5 being most suitable [23]. The pH of the pork steaks after 1 day of marinating was 4.30 for the control, 4.46, 4.82, 5.01 and 5.04 for *L. plantarum* MI-LP1, *Lc. lactis subsp. lactis*, *S. carnosus* and *S. vitulinus*, respectively (Table 1).The first 3 days of fermentation with LAB pH values slightly dropped, but later it raised up ,and after 5 days were 4.73, 4.75, 5.19 and 5.20 for *L. plantarum* MI-LP1, *Lc. lactis subsp. lactis*, *S. carnosus* and *S. vitulinus*, respectively. However, the pH level of the control samples with vinegar acid slightly changed during the marinating period compared with samples inoculated with LAB.

Table 1. The effect of selected lactic acid bacteria on the pH values (means±SE standart error) in marinated pork steaks during storage.

Sample	Duration of storage days		
	1	3	5
	pH		
L. plantarum MI-LP1	4.46±0.03 aA	4.37±0.04 aA	4.73±0.04 aB
L. lactis subsp. *lactis* biovar *diacetylactis* MI-LLd57	4.82±0.05 abA	4.68±0.03 aA	4.75±0.03 aA
S. carnosus MI-SC1	5.01±0.01 bA	4.93±0.05 bA	5.19±0.06 bA
S. vitulinus MI-SV1	5.04±0.03 bA	4.90±0.05 bA	5.20±0.02 bA
Control	4.30±0.06 aA	4.26±0.02 aA	4.37±0.05 aA

Means with different uppercase letters on the same line differ significantly P<0.05 (A-B).
Means with different lowercase letters on the same column differ significantly P<0.05 (a-b), P<0.01 (a-c), P<0.001 (b-c).

3.2. Accumulation of acid and peroxide values in marinated pork steaks

In our study, the highest peroxide value after 3 days of fermentation in marinated pork steaks was measured in samples with S. carnosus, 4.10±0.05 meq/kg of lipid (P=0.03), and the lowest in the samples with *L. plantarum* MI-LP1- 2.89±0.06 meq/kg of lipid (P=0.02) (Table 2). Thuse, the changes in peroxide values in fermented pork steaks indicate that L. plantarum effectively inhibits chain reactions occurring in lipid peroxidation. These results coincide with the findings of Li et al. (Li et al., 2012), who noticed that *L. plantarum* MI -LP1 has antioxidant activity by removing transition metal ions that might otherwise participate in Fenton-type reaction hydroxyl radical-generating reactions.

On the third day of the study, the lowest acid value was determined in samples with selected *L. plantarum* MI-LP1 and *S. vitulinum* (1.58±0.02 and 1.86±0.04 mg KOH per kg of lipid, respectively) (P=0.03).

Table 2. The effect of selected lactic acid bacteria on the acid and peroxide values of fat (means±SE standart error) in marinated pork steaks during storage.

Sample	Duration of storage, days					
	1		3		5	
	Acid value/ (mg/g)	Peroxide value/ (meq/kg)	Acid value/ (mg/g)	Peroxide value (meq/kg)	Acid value/ (mg/g)	Peroxide value (meq/kg)
L. plantarum MI-LP1	1.25±0.02 aA	1.30±0.03 aA	1.58±0.02 aA	2.89±0.06 bB	2.68±0.03 aB	4.11±0.07 aC
L. lactis subsp. *lactis* biovar *diacetylactis* MI-LLd57	1.29±0.03 aA	1.55±0.05 aA	2.30±0.01 bB	3.34±0.04 aB	2.69±0.02 aB	5.07±0.06 bC
S. carnosus MI-SC1	1.30±0.03 aA	1.42±0.02 aA	2.22±0.01 bB	4.10±0.05 aB	2.65±0.03 aB	4.87±0.03 bC
S. vitulinus MI-SV1	1.29±0.01 aA	1.50±0.03 aA	1.86±0.04 aA	3.57±0.03 aB	2.80±0.05 abB	4.80±0.03 bC
Control	1.29±0.04	1.57±0.03	2.15±0.03	3.48±0.01	2.95±0.03	5.10±0.04

aA	aA	bB	aB	aB	bC

Means of the same indicators with different uppercase letters on the same line differ significantly P<0.05 (A-B), P<0.01 (B-C), P<0.001 (A-C).

Means of the same indicators with different lowercase letters on the same column differ significantly P<0.05 (a-b).

3.3. Accumulation of D, L-lactic acid isomers in marinated pork steaks

The results of isomers of lactic acid produced in different samples are shown in Table 3. Production of lactic acid isomers in marinated pork steaks was varied in different strains.   On the third day of the study, the lowest D and the highest L- lactic acid isomers was determined in samples with *L. plantarum* (0.10±0.02) and 5.23±0.09 g/100 g of products, respectively) (P=0.04). *L. plantarum* produces a racemate D, L-lactic acid [24]. Most lactobacilli are D, L-lactic acid producers, but the ratio of the two isomers is slightly variable (Goffin et al., 2005).

Table 3. The effect of selected lactic acid bacteria on the L, D-lactic acid isomers formation (means±SE standart error) in marinated pork steaks during storage.

Sample	Durations of storage, days					
	1		3		5	
	Lactic acid isomers (g/100 g of products)					
	D	L	D	L	D	L
L. plantarum M24I-LP1	0.10±0.01 aA	4.47±0.08 aB	0.10±0.02 aA	5.23±0.09 bB	0.25±0.02 aA	4.86±0.05 bB
L. lactis subsp. lactis biovar *diacetylactis* M28I-LLd57	0.11±0.01 aA	4.35±0.05 aB	0.18±0.01 aA	4.49±0.07 aB	0.25±0.01 aA	4.40±0.06 bB
S. carnosus MI-SC1	0.16±0.02 aA	3.28±0.04 bB	0.25±0.01 cA	3.86±0.08 aB	0.38±0.03 bA	3.72±0.04 aB
S. vitulinus M3I-2 SV1	0.11±0.01 aA	4.25±0.05 aB	0.18±0.03 bA	4.30±0.05 aB	0.30±0.02 bA	4.09±0.06 aB
Control	0.11±0.01 aA	4.39±0.04 aA	0.19±0.01 bB	4.611±0.06 aA	0.26±0.01 aC	4.52±0.07 bA

Means of the same indicators with different uppercase letters on the same line differ significantly *P*<0.05 (A-B), *P*<0.01 (B-C), *P*<0.001 (A-C).

Means of the same indicators with different lowercase letters on the same column differ significantly *P*<0.05 (a-b), *P*<0.01 (b-c), *P*<0.001 (a-c).

3.4. Accumulation of biogenic amines in marinated pork steaks

Cadaverine, tyramine, putrescine, and spermine were influenced by different starter culture in marinated pork steaks fermentation. Tyramine and cadaverine formation started after 1 day of fermentation in marinated pork steaks with *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* MI-LLd57 inoculations. After 1 day of fermentation cadaverine content was significantly higher in sample inoculated with *L. lactis* subsp. *lactis* biovar *diacetylactis* MI-LLd57 compared with all samples at the same fermentation time (*P*=0.0004), (Table 4).

Tyramine is the biogenic amine most generally related to various species LAB and found in fermented sausage which biosynthesizes tyramine [25]. The tyramine toxic level

is 100 to 800 mg/kg [26]. In this research, tyramine contents in marinated pork steaks in-oculated with *Staphylococcus carnosus* LM-SC1 after more 1 day of fermentation were higher than 260 mg/kg (P=0.0003).

Moreover, the accumulation of spermine in marinated pork steaks inoculated with *L. plantarum* MI -LP1 fluctuated, whereas this amine in marinated pork steaks inoculated with *L. lactis* subsp. *Lactis biovar diacetylactis* MI-LLd57 increased statistically significantly after 3 days of fermentation (P=0.0002).

Table 4. The effect of selected lactic bacteria on the biogenic amines formation (means±SE standart error) in marinated pork steaks during storage.

Sample	Biogenic amines (mg/kg)	Duration of storage, days			
		1	3	5	After heat treatment
L. plantarum MI-LP1		<5 aA	<5 aA	<5 aA	<5 aA
L. lactis subsp. *lactis* biovar *diacetylactis* MI-LLd57	Cadaverine	292.26±16.52 cB	312.09±25.18 cB	350.36±27.77 cB	6.32±0.58 aD
S. carnosus MI-SC1		<5 aA	<5 aA	<5 aA	<5 aA
vitulinus MI-SV1		<5 aA	<5 aA	<5 aA	<5 aA
Control		<5 aA	<5 aA	<5 aA	<5 aA
L. plantarum MI-LP1	Tyramine	<5 aA	10.26±1.09 aC	21.28±1.64 bB	26.03±1.53 bB
L. lactis subsp. *Lactis biovar* diacetylactis MI-LLd57		15.09±0.73 aA	36.61±2.55 bB	61.57±4.20 bC	55.25±7.83 bC
S. carnosus MI-SC1		26.64±18.14 bB	57.31±4.57 bA	75.22±2.89 bA	51.97±3.34 bA
S. vitulinus MI-SV1		19.28±1.63 aA	44.07±3.48 bB	69.07±4.77 bC	40.14±3.27 bB
Control		15.26±0.67 aA	23.38±0.92 aA	58.81±1.86 bB	46.33±1.66 bB
L. plantarum MI-LP1	Putrescine	<5 aA	<5 aA	<5 aA	<5 aA
L. lactis subsp. *Lactis biovar* diacetylactis MI-LLd57		59.52±3.92 bA	138.07±12.50 cB	168.11±10.38 cB	10.23±0.70 aC

Sample					
S. carnosus MI-SC1		<5 aA	<5 aA	<5 aA	<5 aA
S. vitulinus MI-SV1		<5 aA	<5 aA	<5 aA	<5 aA
Control		<5 aA	<5 aA	<5 aA	<5 aA
L. plantarum MI-LP1	Spermine	<5 aA	19.38±0.27 bB	<5 aA	55.20±1.59 bB
L. lactis subsp. *Lactis* biovar diacetylactis MI-LLd57		34.28±0.53 bA	108.69±5.18 cB	75.28±1.08 bC	112.01±9.52 cB
S. carnosus MI-SC1		18.16±0.81 aA	64.73±1.82 bB	41.22±1.57 bB	69.44±1.53 bB
S. vitulinus MI-SV1		15.86±0.74 aA	59.46±1.57 bB	45.78±1.42 bB	67.56±2.47 bB
Control		16.21±0.59 aA	56.27±2.11 bB	36.19±0.85 bB	77.06±1.93 bB

Means with different uppercase letters on the same line differ significantly $P<0.05$ (A-B), $P<0.01$ (BC), $P<0.001$ (A-C), $P<0.0001$ (A-D),

Means with different lowercase letters on the same column differ significantly $P<0.05$ (a-b), $P<0.01$ (b-c), $P<0.001$ (a-c).

3.5. Evaluation of color properties in marinated pork steaks

Marinated pork steaks with *S. vitulinus* or *S. carnosus* were darker and redder (lower L* and higher a* values) than control treatment and then samples marinated with *L. plantarum* MI-LP1 and *Lc. Lactis* subsp. *lactis* (Table 5, over fermentation) (*P*=0.03; *P*=0.02). *S. vitulinus* and *S. carnosus* effect on lightness and redness influenced by pH. The redness after grilling was increased in samples with *L. plantarum*, while the nitrite amounts were significant lower in all cases of biofermentation compared with control samples, marinated with 1,5 proc. acetic acid.

Table 5. The effect of selected lactic acid bacteria on the color (means±SE standart error) inmarinated pork steaks during storage.

	Duration of storage, days								
	1			3			5		
Sample	L*	a*	b*	L*	a*	b*	L*	a*	b*
L. plantarum MI-LP1	39.87± 0.37 aB	15.34± 0.49 aA	13.05± 0.61 aA	47.60± 0.13 aB	20.45 ±0.57 aA	16.52± 0.03 aA	45.58 ±0.28 aB	12.87 ±0.11 aA	9.30± 0.02 bC
L. Lactis subsp. *lactis* biovar *diacetylactis* MI-LLd57	37.37± 0.30 aB	17.11± 0.40 aA	13.68± 0.35 aA	45.54± 1.19 aB	19.32 ±0.78 aA	16.52± 0.03 aA	45.58 ±0.28 aB	15.03 ±0.27 bA	9.08± 0.25 bC
S. carnosus MI-SC1	33.77± 0.21	17.11± 0.42	12.26± 0.29	40.08± 0.28	23.64 ±0.53	16.11± 0.27	41.11 ±0.39	14.38 ±0.19	9.86± 0.23

	aB	aA	aA	bB	bB	aA	bC	bA	bC
S. vitulinus MI-SV1	33.81±0.40	16.77±0.24	13.55±0.29	40.27±0.27	23.48±0.67	16.01±0.19	41.50±0.47	16.08±0.19	9.68±0.27
	aB	aA	aA	bB	bA	aA	bB	bA	bC
Control	39.77±0.64	14.42±0.72	12.86±0.29	45.64±0.13	19.45±0.31	18.04±0.08	46.03±0.07	12.84±0.02	10.27±0.04
	aB	aA	aA	aB	aB	bB	aB	aA	bC

Means with different uppercase letters on the same line differ significantly $P<0.01$ (A-B), $P<0.001$ (B-C), $P<0.05$ (A-C).

Means with different lowercase letters on the same column differ significantly $P<0.01$ (a-b).

3.6. Nitrite, nitrate content in grilled pork steaks

The same herbs, for example, oregano and mustard, used for marinating contain nitrates and nitrites which amount can be potentially high. In the control sample content of nitrite decreased from 10,6 mg/kg to 9.22 mg/kg, a total 1.3 mg/kg, and this change was the lowest compared with samples marinated with different lactic acid bacteria, Table 6. Meanwhile, the nitrate content in the control sample increased 2.43±0.01 mg/kg. Nitrite and nitrate were mostly reduced after 5 days of marinating with *L. plantarum* MI-LP1 strain. Nitrites content significantly decreased from 11.41±0.02 mg/kg to 5.5±0.03 mg/kg, nitrates - from14.28±0.03 to 7.76±0.03 mg/kg during 5 days period, when p<0.01 in both cases. The least changes of nitrite and nitrate content were in marinated pork steaks using *L. lactis subsp. lactis biovar diacetylactis* MI-LLd57 strain during 5 days, the decrease was: 1.54±0.01 mg/kg for nitrite and 3.64±0.05 mg/kg for nitrate.

Table 6. The effect of selected lactic acid bacteria on the nitrites and nitrates (means±SE standart error) content in marinated pork steaks during storage.

	Duration of storage, days					
	1		3		5	
	Nitrites (mg/kg)	Nitrates (mg/kg)	Nitrites (mg/kg)	Nitrates (mg/kg)	Nitrites (mg/kg)	Nitrates (mg/kg)
L. plantarum MI-LP1	11.41±0.02 aA	14.28±0.03 aA	9.02±0.02 abA	14.28±0.04 aA	5.5±0.03 bB	7.76±0.03 bB
L. lactis subsp.lactis biovar di-acetylactis MI- LLd57	11.66±0.08 aA	13.9±0.04 aA	10.23±0.19 aA	13.9±0.08 aA	10.12±0.01 aA	10.26±0.09 abA
S. carnosus MI-SC1	11.52±0.02 aA	14.62±0.06 aA	8.48±0.28 bB	14.62±0.11 aA	6.37±0.04 bB	8.32±0.01 bB
Control	10.6±0.02 bA	14.65±0.01 aA	10.04±0.02 aA	14.65±0.09 aA	9.22±0.01 aA	12.22±0.06 aA

Means of the same indicators with different uppercase letters on the same line differ significantly *P*<0.01 (A-B).

Means of the same indicators with different lowercase letters on the same column differ significantly *P*<0.05 (a-b).

3.7. Determination of N-nitrosamine and nitrites, nitrates in grilled pork steaks.

The results show that seven types of detected N-nitrosamines: NDMA, NDEA, NPYR, NDPA, NPIP, NDBA, and NDpheA were reduced in grilled pork steaks after marinating with selected lactic acid bacteria, Table 7. NPIP, NDMA were not determined in those samples, differently than in controls. *L. plantarum* MI-LPI shows a high reducing effect on the formation of all detected N-nitrosamines and nitrite, nitrate content, Table 7. The NDEA values were significantly lower in all samples after biofermentation excepting inoculation with *L. lactis* subsp. *Lactis biovar diacetylactis* MI-LLd57 compared with the control samples (P<0.01).

Table 7. The effect of selected lactic acid bacteria on the N-nitrosamine formation (means of NAs amounts (μg/kg), n = 3) in marinated pork steaks after heat treatment.

Sample	Means of nitrites and nitrates amounts, mg/kg, n= 3		Means of NAs amounts (μg/kg), n = 3							
	Nitrates	Nitrites	NDMA	NDEA	NPYR	NDPA	NPIP	NDBA	NDPheA	Sum of NAs)
L. plantarum MI-LP1	4,32 ± 0.47 c	0,82± 0.12 c	n.d.	n.d.	0.103± 0.010 b	n.d.	n.d.	n.d.	n.d.	0.103± 0.010 c
L. lactis subsp. l*actis* biovar *diacetylactis* MI-LLd57	8.61 ± 0.52 b	2,11± 0.21 a	n.d.	0.437± 0.014 a	0.137± 0.025 b	n.d.	n.d.	n.d.	n.d.	0.574± 0.039 b
S. carnosus MI-SC1	4.93 ± 0.43 c	1,01± 0.35 c	n.d.	n.d.	n.d.	0.213± 0.015 b	n.d.	0.116± 0.031 b	n.d.	0.329± 0.046 b
S. vitulinus MI-SVI	5.91 ± 0.52 b	1,24± 0.63 b	n.d.	0.142± 0.011 b	0.161± 0.022 b	0.221± 0.016 b	n.d.	n.d.	n.d.	0.524± 0.049 b
Control	12.92± 0.22 a	2,22± 0.57 a	0.101± 0.0062 a	0.624± 0.034 a	0.947± 0.029 a	0.371± 0.012 a	0.122 ± 0.014	0.502± 0.041 a	0.741± 0.015	3.185± 0.207 a

Means of the same indicators with different lowercase letters on the same column differ significantly *P*<0.01 (a-b); P<0.001 (a-c).

4. Discussion

The measured pH values of the marinated pork steaks with LAB were higher comparing with the dates of other authors: pH<4 [26], pH<3 [27]. Those differences probably were influenced by the used sugar amount, storage temperature, and other conditions of fermentation. One of the reasons, why the quality of meat can lead to the deterioration is excessive oxidation, the marinating increased acid and peroxide values in all tested

samples. The *L. plantarum* MI-LP1 showed the highest positive effect on hydrolysis and oxidation processes during marinating period of pork steaks (P<0.01). Lin Mei et al. in the study investigated that L. plantarum strains were with antioxidant properties [28].

LAB strains produce lactic acid, which occurs in nature in two optical isomers: D, L-lactic acid. In particular, the isomer L is the only one that can be metabolized by the human, meanwhile the D-lactic acid is toxic for a human organism and can cause serious disease [29]. Thus, in the pork steaks marinade with *L. plantarum* we determined the lowest D (0.10 g/100g of products) and the highest L- lactic acid isomers (5.23 g/100g of products, then P<0.05). These results coincide with Sawitzki et al. [30], who stated that *L. plantarum* strains isolated from fermentation medium produced D, L – lactic acid on the average ratio from 65.28 to 98.84% of L - lactic acid and from 1.16 to 34.7% of D-lactic acid as a final products of sugar fermentation.

Fermented meat can be a source of biogenic amines. Low concentrations of biogenic amines aren't toxic to human health, but high amounts can form carcinogenic nitrosamines [31]. In marinated control pork steaks samples and samples inoculated with *L. plantarum* MI-LP1 strain cadaverine, putrescine, and spermine content were lower than 5 mg/kg throughout all fermentation periods, therefore this represents a safe level. These results related to with Kongkiattikajorn [32] results: accumulation of harmful substances, for example, cadaverine, putrescine, histamine, tryptamine, phenylethylamine, and tyramine in traditional fermented fish sausage with starter culture *L. plantarum* was significantly decreasing. Strains of *L. plantarum* MI-LP1 presented the highest ability to reduce tyramine in culture media, according to Capozzi et al. [33]. Amine oxidase of some indigenous strains of *L. plantarum* due to them be able to degrade spermine [33]. Spermidine can be used by microorganisms as a nitrogen source, so spermidine content in all samples decreased during early fermentation and lower levels were found after 5 days of fermentation [34]. Similar results of biogenic amines production by *S. xylosus* strains were presented by several authors. The effect of *S. carnosus* strain was also positive on the formation of biogenic amine, Casaburi el al., [35] did not find too much biogenic amines production in fermented sausages.

One of the senses that greatly determines a person's choice of meat is color. Changes of color in the surface of meat samples during storage for 1-5 days were monitored by recording the L* (lightness), b* (yellowness) and a* (redness) values. Consumers preference choice meat of more red color, according to Yee Eun Lee [36]. As meat pH increases, the meat becomes darker. Scientific publications state that the main reason for the increasing red color is the addition of *St. carnosus* bacteria which degrades the hydrogen peroxide. As the pH of meat increases with the addition of both *S. vitulinus* or *S. carnosus*, changes in meat color would be expected [37].

Although nitrite and nitrate are not carcinogenic but interact with secondary aliphatic amines and nitrites forme N-nitrosamines which cause health problems such as diabetes, and infectious diseases and may affect aquatic organisms [38]. In our study used oregano and mustard for marinating could have been containing nitrates and nitrites, and the amount of them could be potential high. Nitrite and nitrate were mostly reduced after 5 days of marinating with *L. plantarum* MI-LP1 strain (both decreased two times, P<0.001). These results coincide with Zhu et al. who stated that *L. plantarum* potential to reduce the risk of nitrite [39].

N-nitrosamines in meat products, especially fermented, have raised concerns the safety in meat and their possible carcinogenicity [40]. Acording Xuefei and et.[12] , LAB inoculation has been found to be an effective way to reduce N- nitrosamines substances. The sum of NAs values was significantly lower in the sample with *L. plantarum* (P < 0.001) after biofermentation compared with the control samples. According to Duangjitcharoen [41], *L. plantarum* could be used to protect the bioactivation of N-nitrosamines, as they may play a role in the development of cancer.

In the end, the formation of undesirable compounds during the manufacturing process is unavoidable, however, marinating with LAB, especially with *L. plantarum* could be solution to mitigate these issues.

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