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Research article

Cholinesterase inhibitory activity, antioxidative potential and microbial stability of innovative liver pâté fortified with rosemary extract (*Rosmarinus officinalis*)

Agnieszka Bilska [a], Joanna Kobus-Cisowska [b,*], Dominik Kmiecik [b], Bożena Danyluk [a], Ryszard Kowalski [a], Daria Szymanowska [c], Anna Gramza-Michałowska [b], Oskar Szczepaniak [b]

[a] Department of Meat Technology, Poznań University of Life Sciences, Wojska Polskiego 31/33, 60-624 Poznań, Poland
[b] Department of Gastronomy Science and Functional Foods, Poznań University of Life Sciences, Wojska Polskiego 31/33, 60-624 Poznań, Poland
[c] Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, Wojska Polskiego 48, 60-627 Poznań, Poland

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ABSTRACT

Background: Rosemary (*Rosmarinus officinalis*) contains active substances that have desirable properties for industrial and herbal medicine applications, e.g., essential oils (1.5–2.5%), tannins, flavonoids, triterpenes, saponins, resins, phytosterols, rosmarinic acid and many others. The aim of this study was to determine the influence of rosemary extract and 20% rapeseed oil substitution for animal fat on storage changes and inhibition of cholinesterases in liver pâté.
Results: Preliminary research showed that rosemary extract exhibited antioxidative activity in the system of accelerated Rancimat and Oxidograph tests. Then, rosemary extract was used as an ingredient in liver pâté. During the experiment, meat samples were refrigerated and tested on days 1, 5, 8, 12 and 15 after production. The study proved that the substitution of 20% of animal fat with rapeseed oil decreased the content of saturated acids and increased the content of monoenic fatty acids by approximately 5% and polyene fatty acids by 40%.
Conclusions: In addition to antioxidative activity, the rosemary extract affected the health-promoting value of the samples, which inhibited cholinesterase activity during the entire storage period. The extract inhibited AChE more than BChE.
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1. Introduction

Rosemary (*Rosmarinus officinalis*) is a plant of the *Labiatae* family. It contains active substances that have desirable properties for industrial and herbal medicine applications, e.g. essential oils (1.5–2.5%), tannins, flavonoids, triterpenes, bitters, saponins, resins, phytosterols, rosmarinic acid and many others. Essential oil in rosemary emits strong camphor aroma. It is composed of camphor (14.5%), cineol (12%), borneol (10.5%), pinene (8.5%) and camphene (7%) [1]. Reference publications list a wide range of properties of rosemary resulting from its content of active compounds, with most reports on its antioxidative properties. Researchers prove that the antioxidative effect of rosemary extract could successfully substitute butylhydroxytoluene (BHT) in dehydrated chicken eggs and fish, and rosemary extract could replace it in sausages, pasta, peanut butter and oil [1,2,3].

Scientific reports also provide information about antioxidative and health-promoting properties of compounds contained in rosemary leaves, which inhibit neurodegenerative processes. There were also reports on the influence of rosemary on better results in cognitive tests [4]. Another study confirmed the positive effect of rosemary extract on short-term memory [5]. Rosemary extract exhibited high activity against acetylcholinesterase and butyrylcholinesterase in an experimental combination with cholinesterases [6].

The oxidation of the lipid fraction of meat is an extremely complex process because of the high sensitivity of oxidation products to decomposition, their reactions with other components, the complex effect of catalysts and the presence of antioxidants. These changes are

* Corresponding author.
E-mail address: joanna.kobus@up.poznan.pl (J. Kobus-Cisowska).
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the main cause of limited shelf life of these foodstuffs. The storage of finished products causes emission of undesirable odour and spoils the taste of products. Fat oxidation results in the formation of free radicals, lipid peroxides, and a wide range of other products [7].

Presently, researchers are conducting many investigations, which confirm the fact that a diet rich in plant products helps to keep good health. According to reports in reference publications, the consumption of fruits, vegetables and herbs lowers the incidence of diseases of the metabolic syndrome and neurodegenerative diseases. Therefore, we can observe increased interest in active compounds, including polyphenols, which can be found in rosemary. The inhibition of cholinesterase activity is of key importance in symptomatic treatment of Alzheimer's disease. Cholinesterase inhibitors block these enzymes, increase the content of acetylcholine in cholinergic synapses and improve neurotransmission. Therefore, researchers are continuing investigations on specific natural compounds that might be reversible acetylcholinesterase inhibitors. It is particularly important for improvement of transmission in the cholinergic system of the central nervous system [8].

The aim of the study was to determine the influence of rosemary extract and substitution of 20% of animal fat with rapeseed oil on storage changes in liver pâté and on the activity of pâtés as cholinesterase inhibitors. Liver pâté was selected for research because it is a product with high oxidative instability. It contains a high amount of fat (up to approximately 60%) and non-haeme iron (approximately 30 mg/g of pâté), which is considered the most important pro-oxidant in meat products. Processing such as grinding or mixing of meat increases the oxidative instability, thereby causing interactions between free fatty acids and oxygen in the presence of catalysts such as heat and metalloproteins. The homogeneous structure of liver pâté allows partial exchange of fat with plant oil, and that is why it is possible to enrich the finished meat product with unsaturated fatty acids [9,10,11,12]. In addition, antioxidants, e.g. rosemary, can be used to prolong quality of products and decrease the formation of oxidative changes in fat.

2. Material and methods

2.1. Materials

2.1.1. Rosemary extract

Rosemary extract FORTIUM® R10 DRY (Kemin Food Technologies) was used in the study. It was a beige oil-soluble powder of the following composition: total carbohydrate content, 26.27 g/100 g; total fat content (total lipid fatty acids expressed as triglycerides), 39.59 g/100 g; moisture, 2.75 g/100 g; ash, 31.40 g/100 g; calories, 459.73 kcal; saturated fat (all fatty acids without double bonds), 3.56 g/100 g, monounsaturated fat (all cis-monounsaturated fatty acids), 32.07 g/100 g; polyunsaturated fat (all fatty acids with only cis, cis-methylene-interrupted double bonds), 3.96 g/100 g; cholesterol, 0.00 mg/100 g; sodium, 5.50 mg/100 g; potassium, 4.92 mg/100 g; sugars (all mono- and disaccharides), 0.39 g/100 g; vitamin A, 30.33 IU; calcium, 5.01 mg/100 g; iron, 10.58 mg/100 g.

The dose of the extract used in the study resulted from its content of carnosic acid. In the COMMISSION REGULATION (EU) No. 1129/2011 of 11 November 2011 [13], the dosage of rosemary extract is given as the sum of carnosol and carnosic acid dosages. In the rosemary extract, these substances are the most important ingredients that determine its antioxidant activity. These substances are responsible for the antioxidant properties of preparations as much as 90%. They also show antibacterial, anti-allergic, antiviral and analgesic effects.

2.1.2. Rapeseed oil

'Kujawski' refined rapeseed oil was purchased in a chain store. It was used in Rancimat and Oxidograph tests. Triacylglycerols were isolated from the oil by purification on activated carbon and aluminium oxide. The oil was purified immediately before the experiment. It was dissolved in hexane (1:3 v/v) and passed through a glass column filled with activated carbon, aluminium oxide activated at 300°C, and anhydrous sodium sulphate. During the purification procedure, the column and receptacle were protected from light with aluminium foil. Next, hexane was evaporated in a vacuum evaporator at 40°C. The triacylglycerols obtained in the process were closed in nitrogen atmosphere.

The composition of fatty acids in the refined rapeseed oil was analysed. The results are given in Table 1.

2.1.3. Liver pâté production process

Liver pâtés were produced in the Experimental Processing Plant at the Department of Meat Technology, Poznań University of Life Sciences. The products were produced using the following recipe: class II pork, 43%; finely minced fat, 42%; liver, 15% and broth, 30% in relation to meat and fat ingredients. While mincing a mixture, the following seasonings were added: salt, 1.5%; pepper, 0.15%; marjoram, 0.05% and onion, 0.4%. This composition of ingredients was used as a reference product for experimental variants. There were also pâtés where 20% of animal fat was replaced with rapeseed oil and rosemary extract was added (Table 2).

The finished products were cold-stored at approximately 4°C. Samples were collected for analyses on days 1, 5, 8, 12 and 15 after production. Table 2 shows the chemical composition of the pâtés after production. The basic chemical composition of the control and experimental samples was identified according to ISO standards, i.e. moisture [14], protein [15] (using the Kjeltec-2200 system, Tecator, Sweden), fat [16] (the Soxtec-HT6 system, Tecator). The chloride content was measured by the Volhard method [17].

2.2. Methods

2.2.1. Antioxidative activity of rosemary in lipid systems

A Rancimat apparatus (Metrohm, Switzerland) was used to measure volatile products by conductometry. An oil/triacylglycerol sample (2.5 g) was oxidised with an airstream flowing at a rate of 20 L/h and temperature of 110°C. The end of the induction period was marked by a rapid increase in water conductivity caused by the dissociation of volatile carboxylic acids. The antioxidative efficiency of the extracts was calculated on the basis of induction periods printed by the apparatus. They were used to calculate the value of the protection coefficient (Wo), which is the ratio between the length of the induction period of the sample with an antioxidant and the length of the induction period of the control sample. The amount of oxygen absorbed by the 5-g oil/triacylglycerol sample incubated at 110°C was measured directly in an Oxidograph apparatus (Mikrolab, Denmark). The oxidation resulted in a pressure drop in the reaction vessel, which was recorded using pressure sensors and illustrated as a diagram. The sharp deviation of the curve from which the induction time was read indicated fat oxidation. Like in the Rancimat test, the antioxidative efficiency of the extracts was expressed by means of the protection coefficient (Wo).

2.2.2. Antioxidative activity of rosemary in the pâté system during storage

2.2.2.1. Fatty acids composition. Fatty acids Gas chromatographic analysis of fatty acid methyl esters (AOCS Ce1h-05).

Fat was extracted from the experimental cold cuts according to the procedure described by Folch et al. [19]. A chloroform–methanol mixture (2:1 v/v) was used for extraction.

The fatty acid composition was identified according to AOCS Official method Ce 1 h-05 (AOCS) [18]. Oil samples (10 mg) were dissolved in hexane and transesterified with sodium methylate (0.1 M). Fatty acid methyl esters (FAME) were analysed using an Agilent 7820A GC (Agilent Technologies) equipped with SLB-IL111 capillary columns (Supelco, Bellefonte, PA, USA) (100 m, 0.25 mm and 0.20 μm) and an FID (flame ionisation detector). The initial oven temperature was 150°C, and it was increased to 200°C at 1.5°C/min. The injector and

Table 1
Fatty acid composition [%] of liver pâté, $\bar{x}$ (n = 6) ± s.

Fatty acids	Rapeseed oil	Pork back fat	After production			15-d storage		
			Control	20% RO replacement	20% RO replacement +0.2% RE	Control	20% RO replacement	20% RO replacement +0.2% RE
C 12:0	0.00	0.12 ± 0.02	0.05 ± 0.02	0.05 ± 0.00	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
C 14:0	0.00	1.80 ± 0.02	1.24 ± 0.03	1.05 ± 0.04	1.03 ± 0.03	1.20 ± 0.04	1.07 ± 0.04	1.03 ± 0.08
C16:0	4.44 ± 0.04	25.89 ± 0.08	24.27 ± 1.40	20.87 ± 0.98	20.73 ± 1.31	23.87 ± 0.96	20.57 ± 1.11	21.11 ± 1.12
C16:1	0.20 ± 0.00	2.73 ± 0.04	2.26 ± 0.29	1.85 ± 0.15	1.91 ± 0.18	2.33 ± 0.32	2.07 ± 0.27	1.89 ± 0.18
C18:0	1.58 ± 0.02	13.98 ± 0.06	14.36 ± 1.52	12.33 ± 1.19	12.50 ± 1.50	14.26 ± 0.97	11.94 ± 0.79	12.78 ± 1.31
C18:1	61.95 ± 0.02	45.91 ± 0.09	45.27 ± 2.04	48.33 ± 1.24	48.21 ± 1.94	45.69 ± 1.18	48.73 ± 1.19	47.76 ± 1.52
C18:2	19.26 ± 0.00	7.63 ± 0.04	9.29 ± 0.36	11.78 ± 0.38	11.74 ± 0.43	9.36 ± 0.27	11.74 ± 0.19	11.61 ± 0.34
C18:3	11.30 ± 0.00	1.61 ± 0.02	1.57 ± 0.09	3.04 ± 0.36	3.02 ± 0.21	1.55 ± 0.10	3.15 ± 0.19	3.06 ± 0.37
C20:0	0.55 ± 0.00	0.19 ± 0.01	0.17 ± 0.02	0.22 ± 0.03	0.25 ± 0.02	0.19 ± 0.03	0.22 ± 0.01	0.25 ± 0.04
C20:1	0.21 ± 0.00	0.00	0.20 ± 0.00	0.20 ± 0.02	0.25 ± 0.00	0.18 ± 0.00	0.20 ± 0.01	0.20 ± 0.02
C22:0	0.32 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C22:1	0.21 ± 0.00	0.17 ± 0.01	0.33 ± 0.17	0.29 ± 0.07	0.31 ± 0.08	0.32 ± 0.13	0.26 ± 0.08	0.26 ± 0.08
∑SFA	6.88 ± 1.89	41.96 ± 11.36	41.09 ± 10.90	34.51 ± 9.36	34.57 ± 9.33	40.57 ± 10.74	33.86 ± 9.19	35.23 ± 9.51
∑MUFA	62.56 ± 30.87	48.81 ± 25.70	48.06 ± 25.35	50.66 ± 27.23	50.67 ± 27.13	48.52 ± 25.58	51.25 ± 27.42	50.11 ± 26.91
∑PUFA	30.56 ± 5.63	9.24 ± 4.26	10.85 ± 6.17	14.82 ± 6.18	14.76 ± 6.17	10.91 ± 6.23	14.89 ± 6.08	14.67 ± 6.04
PUFA/SFA	4.44	0.22	0.26	0.43	0.43	0.27	0.44	0.42
n6/n3	1.70	4.75	5.93	3.87	3.89	6.03	3.73	3.79

SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids (PUFA n-6/n-3 – from n-6/n-3 family); $\bar{x}$: mean value; n: number of replications; s: standard deviation.

detector temperature was 250°C and split 1:10. The carrier gas was helium at 1 mL/min. FAME were identified by comparing them with commercially available standards – grain fatty acid methyl ester mix (Supelco, Bellefonte, PA, USA). The results were expressed as percentage of the total fatty acids.

2.2.2.2. Peroxide value (PV). Peroxide value (PV) was measured according to the ISO standard [20] in extracted lipid fraction according to the Folch procedure [19] (chloroform:methanol solvent system at a ratio of 2:1, *v*/v). The results were expressed as meq. O_2 kg^{-1}.

2.2.2.3. Malondialdehyde content (TBARS). The TBARS (2-thiobarbituric acid reactive substances) value was measured by the Tarladgis distillation method [21] modified by Pikul et al. [22]. The results were expressed as mg malondialdehyde (MDA) kg^{-1}.

2.2.2.4. DPPH scavenging capacity. Free radical scavenging capacity was assessed by a test with alcohol solution of stable radical DPPH at a wavelength of λ = 517 nm according to the modified method developed by Brand-Wiliams et al. [23], which used DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma). The results were shown as variation in inhibition, which was the ratio between the absorbance of the solution under analysis and absorbance of the zero sample multiplied by 100%.

Table 2
Liver pâté formulations [g/kg] and their basic composition.

Sample	Control	20% RO replacement	20% RO replacement +0.2% RE
Liver pâté formulations [g kg^{-1}]			
Pork meat (class II)	430	430	430
Pork back fat	420	336	336
Rapeseed oil	–	84	84
Pork liver	150	150	150
Mix of spices	21	21	21
Rosemary extract	–	–	2
Basic composition of pâtés [%] (n = 4)			
Protein	9.20 ± 0.12	9.16 ± 0.08	9.13 ± 0.04
Fat	42.19 ± 0.26	44.19 ± 0.06	44.22 ± 0.16
Water	46.15 ± 0.29	44.93 ± 0.35	44.79 ± 0.49
Salt	1.57 ± 0.02	1.65 ± 0.03	1.69 ± 0.01

2.2.2.5. pH and water activity (a_w) measurement. Active acidity (pH) was assessed by the method meeting the ISO standard [24]. Water activity (a_w) was measured with an AquaLab series 4TE apparatus (Pullman, USA), following the guidelines in the instruction manual.

2.2.2.6. Microbial stability. The total count of microorganisms was measured according to ISO 4833-1:2013 [25]. The count of enterococci was measured according to standard PN-A-82055-7:1997 [26] on the Slanetz and Bartley Medium. The rods of the Enterobacteriaceae family were isolated and counted on a Violet Red Bile Glucose agar – VRGB [27, 28]. Bacteria of the genus *Pseudomonas* were detected, isolated and counted on a cephaloridine fucidin cetrimide (CFC) agar [29].

2.2.3. Rosemary extract activity as a cholinesterase (ChE) inhibitor in liver pâté

Cholinesterase (ChE) inhibition was measured in aqueous extracts obtained from defatted liver pâté. The inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was measured by the modified spectrometric method developed by Ellman et al. [30]. A POLARstar Omega Plate Reader (BMG LABTECH) with 96-well plates of the maximum capacity of 300 µl was used for measurements. Acetylcholine/butyrylcholine hydrolysis resulted in a colour change of the enzymes under study, which was observed during absorbance measurements made at a wavelength of 412 nm, 10 min after pipetting on a microplate.

Reagent solutions were prepared in Tris–HCl buffer (50 mmol/dm^3, pH 8). The enzyme solutions were prepared by dissolving 2 U/ml in 2 ml of phosphate buffer. The reaction mixture was composed of 0.035 cm^3 of the sample under analysis, 0.086 cm^3 of Tris–HCl buffer (50 mmol/dm^3, pH 8), 0.035 cm^3 of ATChI or BTCh (1.5 mmol/dm^3), 0.194 cm^3 of DTNB (0.3 mmol/dm^3 with 10 mmol/dm^3 NaCl and 2 mmol/dm^3 $MgCl_2 \times 6\ H_2O$) and AChE or BChE solution. The absorbance was measured after 15 min (BChE) or 30 min (AChE). At the same time, the positive control sample containing the known ChE inhibitor, i.e., eserine (90.7 µmol × dm^3), and the negative control sample without the ChE inhibitor were analysed. The activity was calculated by generating eserine modelling curves within the following concentration ranges: 0.08 µmol/dm^3–6.50 µmol/dm^3 (AChE) and 0.08 µmol/dm^3–8.30 µmol/dm^3 (BChE). All the samples were analysed in eight replicates. The results were expressed as inhibition percentage.

2.3. Statistical analysis

The experiment was repeated three times. All measurements were duplicated. The results were analysed statistically with Statistica 13.1 and Excel 2007 software. The data were expressed as mean ± standard deviation of independent measurements for four samples ($n = 6$). The effect of treatment (replacement of animal fat with flaxseed oil with or without the plant extract additive and the control sample without the replacement and additive $L = 6$) and storage time (days, $L = 5$) were analysed. Analysis of variance (ANOVA) was carried out for a CRD (completely randomised design) experiment. Then, Tukey's test was applied to compare the means at a significance level of $P \leq 0.05$. The slope of the regression curve (coefficient A/24 h) was analysed to compare the dynamics of lipid oxidation and hydrolytic changes during storage.

3. Results and discussion

3.1. Rosemary antioxidative activity in lipid systems

The experiment showed that the rosemary extracts exhibited antioxidative activity in the systems with rapeseed oil and rapeseed oil triacylglycerols. The Oxidograph test showed that the protection coefficient of 1000 ppm of the rosemary extract was not significantly different from the sample with BHT. The analysis showed that 500, 700 and 1000 ppm of the rosemary extract added to oil and triacylglycerols increased the protection coefficient values, which ranged from 1.16 to 1.54. These findings were also confirmed in the Rancimat test, where the protection coefficient values of the samples including 500–1000 ppm of rosemary extract ranged from 1.21 to 1.76. The effect of active compounds in the rosemary extract on extension of the induction period in a Rancimat test was studied by Yang et al. [31], who proved that rosemary extract (200 ppm) added to rice, cotton and soy oils exhibited antioxidative activity comparable to that of synthetic BHA and BHT antioxidants. Upadhyay et al. [32] conducted a Rancimat test, which showed that rosemary extract could be used to stabilise sunflower fat during deep-frying. Upadhyay and Mishra [33] observed that the rosemary extract could be used for frying within a wide range of temperatures. The antioxidative capacity of rosemary extract was attributed to the presence of phenolic diterpenes, which scavenged singlet oxygen, hydroxyl radicals and lipid peroxyl radicals, thus preventing lipid oxidation [34].

Antioxidants are used to extend the shelf-life and delay oxidative changes, especially in fats. The research findings let us conclude that rosemary extract applied to products containing rapeseed oil could delay oxidative changes in the fat. Simultaneously, these results predict the positive influence of rosemary extract on the inhibition of changes in the pâté fat, where some of the animal fat is replaced with rapeseed oil. However, it is difficult to assess this effect at this stage of the research because, in contrast to accelerated tests, extracts exhibit different activity in emulsion systems (e.g. in meat products) than in vegetable oil on its own. This is because in emulsion systems, these compounds are absorbed at the phase limits [35,36].

3.2. The effect of rosemary on changes in the shelf life of experimental pâtés

3.2.1. Fatty acid composition

Rapeseed oil is one of the most valuable vegetable oils. It is mostly composed of monounsaturated fatty acids, mainly oleic acid, followed by polyunsaturated fatty acids, including linoleic acid (LA), which belongs to the group of omega-6 acids, and alpha-linolenic acid (ALA), which belongs to the omega-3 family. Saturated fatty acids, mostly palmitic acid, have the smallest share in rapeseed oil [37]. The cold-pressed rapeseed oil used in this study contained (Table 1) 6.88% of SFA; 62.56% of MUFA, mostly oleic acid (18:1–61.95%) and 30.56% of PUFA. The ratio between n–6 and n–3 acids amounted to 1.7:1.

The amount of saturated acids in the total content of fatty acids in the control sample was 41.09%. The amount of saturated acids in the samples with rapeseed oil substituted for 20% of animal fat was 34.57% (Table 1). Palmitic acid C16:0 was the most abundant (20.57–25.27%). Oleic acid C18:1 was the predominant monounsaturated fatty acid (45.27–48.73%).

The analysis of the composition of the offal cold cuts immediately after their production (Table 1) showed that the replacement of 20% of animal fat with rapeseed oil resulted in positive nutritional changes in the proportions of individual groups of fatty acids. The share of PUFA in the total content of fatty acids increased from approximately 11% in the control sample to approximately 15% in the samples where animal fat had been replaced. The amount of SFA decreased. The amount of α-linolenic acid increased 2.5 times. Consequently, the PUFA n–6:n–3 ratio improved and amounted to 6:1 in the control sample and 3.9:1 in the substitution samples. According to nutritional recommendations, the normal ratio between these acids in the daily diet of a healthy person should range from 4:1 to 2:1 [38].

The 15-d storage of the samples did not cause changes ($P > 0.05$) in the composition of fatty acids in individual experimental cold cuts.

3.2.2. Changes in lipids

The oxidative changes of lipids in the experimental cold cuts were checked by monitoring changes in the content of primary (peroxide value – PV) and secondary products of oxidation (TBARS) during cold storage.

The analysis showed a statistically significant increase in the peroxide value in the control sample and the one with rapeseed oil substituted for 20% of animal fat until the 12th day after the production (Table 3). Further storage resulted in a statistically significant decrease in this parameter. The sample with rapeseed oil substituted for 20% of animal fat was characterised by lower increase in the peroxide value during the whole period of storage. Zeng et al. [7] observed a similar dependency in their research. They found that samples of Cantonese sausage with natural antioxidants were characterised by lower peroxide value than the control sample.

According to many authors, the rate of oxidative changes occurring in fat is determined by various factors, e.g. the composition of fatty acids, the presence of pro-oxidants and antioxidants and storage conditions [7,12]. TBARS is the most common method for monitoring lipid oxidation in meat and meat products. During the whole storage period, the highest TBARS content was observed in the control sample. Similar to changes in the peroxide value, during product storage, secondary products of lipid oxidation in the samples with rapeseed oil substituted for 20% of animal fat tended to increase less dynamically than that in the control sample. The addition of rosemary extract slowed down the formation of secondary metabolites of lipid oxidation (Table 3). The authors of studies on pâtés with sage and rosemary oils made similar observations. They found that rosemary oil inhibited the growth of TBARS [12]. Other authors observed that the addition of natural antioxidants such as rosemary extract successfully slowed down fat oxidation, and their effect was similar to that of synthetic antioxidants [39]. Some authors described their experiments on rosemary, which was added to meat products (pork sausages and pâtés) to stabilise fat [40,41].

The addition of an antioxidant to the sample decreases absorbance by reducing the amount of radicals. The larger the amount of the reduced radical is, the higher the potential of the sample is [42]. The samples used in our experiment exhibited minimal but statistically significant capacity to bind DPPH free radicals. Between days 1 and 15 after production, the highest changes were observed in the control sample (Table 3).

3.2.3. Changes in physical determinants

The pH value is measured to assess the quality and shelf life of meat and meat products. The pH value of a meat product affects various qualitative factors such as colour, consistency and taste [43]. It enables

Table 3
Influence of time on changes in fats in the experimental cold cuts $\bar{x}$ (n = 6) ± s.

Sample	Storage time (d)					Dynamics of changes*	
	1	5	8	11	15	Coefficient Ax10^{-2}/24 h	R^2
Peroxide value [mEq O_2/kg]							
Control	$0.15^{bc} \pm 0.01$	$0.17^{d} \pm 0.01$	$0.19^{f} \pm 0.01$	$0.23^{h} \pm 0.02$	$0.15^{bc} \pm 0.01$	0.20	0.08
20% RO replacement	$0.14^{ab} \pm 0.02$	$0.16^{cd} \pm 0.01$	$0.17^{de} \pm 0.01$	$0.21^{g} \pm 0.01$	$0.17^{d} \pm 0.01$	0.30	0.44
20% RO replacement + 0.2% RE	$0.13^{a} \pm 0.01$	$0.15^{bc} \pm 0.01$	$0.16^{bcd} \pm 0.01$	$0.17^{de} \pm 0.01$	$0.19^{ef} \pm 0.01$	0.40	0.99
TBARS [mg MDA/kg]							
Control	$1.18^{de} \pm 0.02$	$1.30^{fg} \pm 0.03$	$1.52^{i} \pm 0.05$	$1.62^{j} \pm 0.02$	$1.84^{l} \pm 0.08$	4.70	0.97
20% RO replacement	$0.98^{b} \pm 0.03$	$1.23^{ef} \pm 0.04$	$1.35^{g} \pm 0.07$	$1.51^{i} \pm 0.01$	$1.73^{k} \pm 0.08$	5.10	0.99
20% RO replacement + 0.2% RE	$0.89^{a} \pm 0.03$	$1.05^{c} \pm 0.07$	$1.13^{cd} \pm 0.03$	$1.21^{e} \pm 0.03$	$1.43^{h} \pm 0.10$	3.50	0.96
DPPH [inhibition percentage]							
Control	$32.04^{i} \pm 0.22$	$31.75^{i} \pm 0.53$	$30.76^{h} \pm 1.03$	$28.12^{g} \pm 0.63$	$23.81^{a} \pm 0.37$	−56.7	0.84
20% RO replacement	$30.68^{h} \pm 1.37$	$27.44^{fg} \pm 0.78$	$26.92^{def} \pm 0.50$	$26.12^{cd} \pm 1.06$	$24.94^{b} \pm 1.28$	−36.80	0.90
20% RO replacement + 0.2% RE	$31.27^{hi} \pm 1.29$	$27.38^{efg} \pm 0.58$	$27.12^{ef} \pm 0.92$	$26.42^{cde} \pm 0.40$	$25.65^{bc} \pm 0.39$	−35.30	0.81

$\bar{x}$: mean value; n: number of replications; s: standard deviation; a. b…: mean values marked with different letters differ significantly according to Tukey's test ($P \leq 0.05$).
* Linear regression equation: y = Ax + B (y: dependent variable; x: independent variable; A: independent variable coefficient per slope of the line; B: intercept), coefficient A/24 h: change of coefficient A during 24 h storage; R^2: coefficient of determination, $P < 0.05$.

the identification of meat defects (PSE and DFD) and indication of hydrolytic changes of fat in the product. The experimental cold cuts were characterised by a similar course of pH variation during the 15-d storage (Table 4). Apart from that, the control sample was characterised by slightly higher pH values than the samples with rapeseed oil substituted for 20% of animal fat.

Water activity affects a wide range of determinants, including the product's susceptibility to decay. Monitoring the optimal water activity for a given product gives a possibility to obtain a product of the highest quality and maximum shelf-life and to minimise the content of preservatives [44]. Water activity ranged from 0.94 (in the control sample on day 15 after production) to 0.97 (in the samples with rapeseed oil substituted for 20% of animal fat immediately after production). The lowest variation in water activity during storage was observed in the sample containing rosemary extract and rapeseed oil substituted for 20% of animal fat (Table 4).

3.2.4. Microbial stability

The investigations showed that the content of aerobic microorganisms in the samples did not exceed 3.66 $\log_{10}$ cfu g^{-1} during the 15-d storage (Table 4). *Pseudomonas* bacteria are part of the saprophytic microflora causing the decay of cold-stored meat products, which have access to oxygen. They are capable of producing extracellular enzymes, i.e. lipases and proteinases at low temperature. When the count of microorganisms exceeds 10^7–10^9 cfu/g, these enzymes cause irreversible changes in meat quality and unacceptable smell [45]. In our study, the count of *Pseudomonas* bacteria ranged from 10^2 to 10^3 cfu/g.

Enterococci can survive fermentation, pasteurisation and cooking during food processing [46,47,48]. Many authors think that enterococci are good indicators of food production hygiene because they are temperature resistant [46,49] and they may cause food decay themselves [47,50,51]. We did not observe temperature-resistant enterococci in our study during the 15-d storage. Like in the study on vacuum-packaged pâtés stored at 4°C for 28 d, we did not observe bacteria of the Enterobacteriaceae family in our experiment [10].

3.3. Anti-neurodegenerative properties of pâtés

The study also included assessment of the influence of the rosemary extract additive on expected health-promoting properties of pâtés, i.e. the capacity to inhibit the activity of cholinesterases – acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Table 5

Table 4
Variation of physical determinants and the microbial quality in the experimental cold cuts during storage $\bar{x}$ (n = 6) ± s.

Sample	Storage time (d)					Dynamics of changes*	
	1	5	8	11	15	Coefficient Ax10–2/24 h	(R2)
pH							
Control	$6.36^{abcde} \pm 0.07$	$6.42^{bcde} \pm 0.03$	$6.51^{ef} \pm 0.09$	$6.58^{f} \pm 0.11$	$6.46^{def} \pm 0.24$	1.10	0.50
20% RO replacement	$6.27^{a} \pm 0.01$	$6.31^{abc} \pm 0.06$	$6.39^{abcde} \pm 0.03$	$6.46^{def} \pm 0.12$	$6.44^{cdef} \pm 0.15$	1.40	0.90
20% RO replacement + 0.2% RE	$6.30^{ab} \pm 0.06$	$6.31^{abc} \pm 0.11$	$6.39^{abcde} \pm 0.02$	$6.40^{abcde} \pm 0.02$	$6.31^{abc} \pm 0.13$	0.40	0.15
a_w							
Control	$0.96^{fg} \pm 0.01$	$0.96^{ef} \pm 0.01$	$0.96^{ef} \pm 0.01$	$0.95^{bc} \pm 0.00$	$0.94^{a} \pm 0.00$	−0.20	0.83
20% RO replacement	$0.97^{gh} \pm 0.00$	$0.96^{ef} \pm 0.00$	$0.96^{def} \pm 0.00$	$0.96^{cd} \pm 0.00$	$0.95^{b} \pm 0.00$	−0.10	0.98
20% RO replacement + 0.2% RE	$0.97^{h} \pm 0.00$	$0.96^{ef} \pm 0.00$	$0.96^{de} \pm 0.00$	$0.96^{de} \pm 0.00$	$0.96^{cd} \pm 0.00$	−0.10	0.85
Total count of microorganisms [$\log_{10}$ cfu/g]							
Control	$2.79^{a} \pm 0.05$	$3.21^{e} \pm 0.03$	$3.26^{f} \pm 0.02$	$3.40^{g} \pm 0.03$	$3.62^{i} \pm 0.05$	5.30	0.93
20% RO replacement	$2.78^{a} \pm 0.06$	$3.09^{c} \pm 0.03$	$3.29^{f} \pm 0.03$	$3.37^{g} \pm 0.02$	$3.66^{i} \pm 0.03$	5.80	0.96
20% RO replacement + 0.2% RE	$2.88^{b} \pm 0.02$	$3.14^{d} \pm 0.02$	$3.29^{f} \pm 0.02$	$3.39^{g} \pm 0.02$	$3.49^{h} \pm 0.01$	4.20	0.96
Pseudomonas [$\log_{10}$ cfu/g]							
Control	$2.70^{b} \pm 0.03$	$2.79^{c} \pm 0.07$	$3.10^{e} \pm 0.06$	$3.32^{g} \pm 0.02$	$3.69^{i} \pm 0.01$	7.10	0.95
20% RO replacement	$2.76^{bc} \pm 0.07$	$3.08^{e} \pm 0.03$	$3.15^{e} \pm 0.03$	$3.23^{f} \pm 0.02$	$3.45^{h} \pm 0.03$	4.40	0.93
20% RO replacement + 0.2% RE	$2.60^{a} \pm 0.09$	$2.88^{d} \pm 0.06$	$3.13^{e} \pm 0.04$	$3.24^{f} \pm 0.05$	$3.45^{h} \pm 0.04$	5.9	0.98

$\bar{x}$: mean value; n: number of replications; s: standard deviation; a. b …: mean values marked with different letters show significantly difference according to Tukey's test ($P \leq 0.05$).
* Linear regression equation: y = Ax + B (y: dependent variable; x: independent variable; A: independent variable coefficient per slope of the line; B: intercept), coefficient A/24 h: change of coefficient A during 24 h-storage; R^2: coefficient of determination, $P < 0.05$.

Table 5
Acetylcholinesterase and butyrylcholinesterase inhibitory activity of rosemary extracts in liver pâté $\bar{x}$ (n = 6) ± s.

Sample	Storage time					Dynamics of changes*	
	Day 1	Day 5	Day 8	Day 12	Day 15	Coefficient Ax10^{-2}/24 h	(R^2)
AChE (μM eserine/100 g d.m.)							
Control	$6.12^{b} \pm 0.54$	$5.80^{a} \pm 0.27$	$5.79^{a} \pm 0.22$	$5.88^{a} \pm 0.19$	$5.78^{a} \pm 0.33$	−0.06	45.27
20% RO replacement	$6.31^{b} \pm 0.55$	$6.65^{b,A} \pm 0.31$	$5.95^{a} \pm 0.27$	$6.23^{b} \pm 0.41$	$6.47^{b} \pm 0.21$	−0.01	0.16
20% RO replacement + 0.2% RE	$7.35^{c} \pm 0.35$	$7.88^{d} \pm 0.12$	$7.57^{c} \pm 0.29$	$7.58^{c} \pm 0.29$	$7.46^{c} \pm 0.31$	−0.01	0.07
BChE (μM eserine/100 g d.m.)							
Control	$0.23^{a} \pm 0.02$	$0.30^{a} \pm 0.02$	$0.26^{a} \pm 0.01$	$0.24^{a} \pm 0.01$	$0.24^{a} \pm 0.01$	0.00	4.01
20% RO replacement	$0.21^{a} \pm 0.01$	$0.29^{a} \pm 0.08$	$0.27^{a} \pm 0.01$	$0.25^{a} \pm 0.01$	$0.25^{a} \pm 0.00$	0.00	5.63
20% RO replacement + 0.2% RE	$1.19^{b} \pm 0.07$	$1.13^{b} \pm 0.09$	$1.26^{b} \pm 0.01$	$1.03^{b} \pm 0.00$	$0.89^{b} \pm 0.00$	−0.07	59.43

$\bar{x}$: mean value; n; number of replications; s; standard deviation; a. b …: mean values marked with different letters show significant difference according to Tukey's test ($P \leq 0.05$).
* Linear regression equation: y = Ax + B (y: dependent variable; x: independent variable; A: independent variable coefficient per slope of the line; B: intercept), coefficient A/24 h: change of coefficient A during 24 h storage; R^2: coefficient of determination, $P < 0.05$.

shows the results of the analysis. The cholinesterase inhibitory capacity was expressed as eserine equivalents.

The replacement of animal fat with rapeseed oil had no effect on activity against cholinesterases. The rosemary extract additive significantly increased the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The samples exhibited activity against ChE during the whole storage period. The rosemary extract added to the pâté inhibited AChE more than BChE.

The inhibition of cholinesterase activity is of key significance to symptomatically treatment Alzheimer's disease. Cholinesterase inhibitors increase the amount of acetylcholine in cholinergic synapses, which improves neurotransmission. Therefore, researchers are conducting numerous studies on specific natural compounds, which can be reversible esterase inhibitors. It is important to improve the transmission in the cholinergic system of the central nervous system [8,52,53]. There are elaborate descriptions of the activity of phytochemicals as cholinesterase inhibitors. However, there have been few reports on the effect of rosemary compounds in this aspect. Earlier research on mice showed the positive effect of rosemary leaf brew against BChE and AChE [54]. Ozarowski et al. [6] conducted a study on rats and observed a similar effect of rosemary leaf extract as a ChE inhibitor. Molecular modelling of rosemary compounds showed that some of the main components of rosemary may exhibit activity against ChE. This partly explains the anticholinesterase effect observed in pâté extracts [54].

The study proved that apart from the antioxidative activity, rosemary had influence on ChE inhibition. This activity was stable during the 15-d storage of liver pâté meat products.

3.4. Principal component analysis (PCA)

PCA was applied to observe possible clusters in the pâté samples under analysis. The first two principal factors accounted for 83.85% (PF1 = 58.76% and PF2 = 25.76%) of the total variation. The PCA results showed noticeable differences between the control samples and the products prepared with rapeseed oil and those containing rapeseed oil and rosemary extract (Fig. 1).

Factor 1 was mainly correlated with the TBARS (r = 0.970), the content of *Pseudomonas* bacteria (r = 0.913) and the total count of mesophilic aerobic bacteria (r = 0.893). It was negatively correlated with the water activity (r = −0.924). Factor 2 was mainly negatively correlated with the inhibition of AChE (r = −0.854) and BChE (r = −0.789). The control pâté samples and samples of pâté enriched with rapeseed oil are taken as the X-axis. Sample 5 (the control sample after 15 d of storage) and sample 10 (pâté with rapeseed oil after 15 d of storage) are two exceptions. These two samples are taken as the X axis, and they were characterised by the highest content of *Pseudomonas* and mesophilic aerobic bacteria and the lowest DPPH activity. All samples enriched with rapeseed oil and rosemary extract are taken as the X axis. They were characterised by the highest

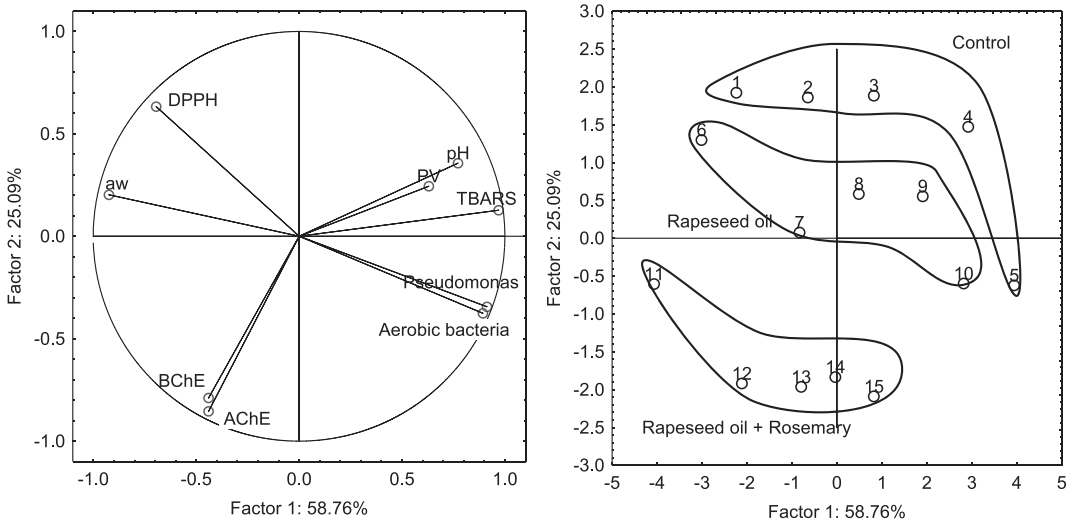

Fig. 1. Principal component analysis (PCA) of the loading plot and the score plot of data referring to the peroxide value (PV), TBARS, DPPH, pH, water activity (aw), total content of *Pseudomonas* and mesophilic aerobic bacteria and inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

inhibition of AChE and BChE in comparison with the other two products (the control pâté and the pâté with rapeseed oil only).

4. Conclusion

The research showed that partial substitution of animal fat with rapeseed oil influenced the composition of the lipid fraction of liver pâté. It reduced the content of saturated fatty acids but increased the content of mono- and polyunsaturated fatty acids. These changes are nutritionally favourable, and they result in a product enriched with omega-3 fatty acids. The research showed that the rosemary extract additive successfully reduced lipid oxidation during the cold storage of liver pâtés with 20% of animal fat substituted with rapeseed oil. The rosemary extract also increased the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

The research findings can be used to create a new group of storage-stable meat products of higher nutritional value by enriching them with oils containing large amounts of unsaturated fatty acids.

Declarations of interest

None.

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