Dry Fermented Sausages with Total Replacement of Fat by Extra Virgin Olive Oil Emulsion and Indigenous Lactic Acid Bacteria

Running title: Sausages with total replacement of fat

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SUMMARY

Research background. Formulations based on vegetable or fish oil and modifications in the production technology of dry fermented sausages have emerged in recent years aiming to achieve the desirable target of reducing the fat content of these meat products. However, previous efforts have confronted many difficulties, such as high weight loss and unacceptable appearance due to intensely wrinkled surfaces and case hardening. The objective of this study was to produce and evaluate dry fermented sausages by utilizing a meat protein-olive oil emulsion as fat substitute and indigenous lactic acid bacteria (LAB) with probiotic properties isolated from traditional Greek meat products.

Experimental approach. A novel formulation with extra virgin olive oil and turkey protein was developed to totally replace the conventionally added pork fat. Identification and evaluation of the probiotic and safety characteristics of autochthonous LAB isolates from spontaneously fermented sausages were performed and three LAB isolates were finally selected as starter cultures. Physicochemical, microbiological and sensory analyses were

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carried out in all treatments (control, L. acidophilus, L. casei, L. sakei, P. pediococcus) during fermentation.

Results and conclusions. Ready-to-eat sausages were found to be microbiologically stable. The olive oil-based formulation produced in this study generated a mosaic pattern visible in the sliced product simulating the fat in conventional fermented sausages and was regarded as an ideal fat substitute for the production of fermented sausages. An autochthonous isolate of Lactobacillus casei exhibited the best adaptation in the final products as it was molecularly identified to be present in the highest counts among the LAB isolates used as starter cultures.

Novelty and scientific contribution. A novel and high-quality dry fermented meat product was produced replacing added pork fat with a fat substitute based on a meat protein-olive oil emulsion. Autochthonous LAB with in vitro probiotic properties could have a potential use in large-scale novel dry fermented sausages production. Such isolates could be used as starters in an effort to standardize the production process and retain the typical organoleptic and sensory characteristics. Moreover, isolates like L. casei 62 that survived in high counts in the final products, can increase the safety of fermented sausages by competing not only with pathogens but also with the indigenous microbiota and could have a potential functional value for the consumer.

Key words: fat substitute, probiotics, fermented meat products

INTRODUCTION

Dry fermented sausages are widely consumed meat products with special sensory characteristics such as exceptional taste and distinctive flavour (1). However, conventional dry fermented sausages cannot be classified as “healthy” foods according to the modern nutritional trends that praise the benefits of low-fat diets since their fat concentration is high (30.0-50.0 g fat per 100 g) and rich in saturated fatty acids (2). High animal fat intake is regarded to contribute to the prevalence of the modern nutrition-related health problems such as heart disease, hypertension, obesity, diabetes and cancer (3). Therefore, WHO (3) and USDA (4) have proposed to limit the daily fat intake to less than 30.0 % of total calories and to adapt a Mediterranean type of diet that includes higher consumption of olive oil that is rich in unsaturated fatty acids.

New formulations based on vegetable or fish oil and modifications in the production technology of dry fermented sausages have emerged in order to achieve the desirable target
of reducing the fat content of these meat products (2,5). However, the anticipated reduction or replacement of fat content cannot be infinite in these products since fat plays an important technological role by contributing to the continuous loss of water and movement of moisture outwards, procedures with positive impact to the fermentation process. Moreover, fat contributes to various properties of the final product such as flavour, texture, mouthfeel, juiciness and lubricity (6). No data are available as regards the achievement of totally replacing the added animal fat in fermented sausages. Previous efforts towards reducing the fat content of dry fermented sausages confronted many difficulties. In particular, compared to their ordinary counterparts, dry fermented sausages with low fat content (more than 30.0 % reduction) appeared harder, with higher weight loss and unacceptable appearance due to the intensely wrinkled surfaces and case hardening (2,6,7).

The use of starter cultures in dry sausage production is deemed necessary in order to control the manufacturing process, attain the standardized sensory properties and guarantee the microbiological safety of the final product. Starter cultures may exhibit in vitro probiotic properties such as growth in low pH concentrations, tolerance in different concentrations of bile salts and antimicrobial activity against pathogens. Nevertheless, the in vivo functional value of viable (>6.0 log CFU/g) probiotic cultures in foodstuffs should entail clinical-based evidence of specific beneficial effects to human health such as desirable modulation of intestinal flora, prevention of diarrhoea, improvement of constipation symptoms, prevention of allergies and reduction of plasma cholesterol levels (8,9). Commercial probiotic cultures of lactic acid bacteria (LAB) used as starter cultures in industrial production of dry fermented sausages are of milk product origin (10). However, non-commercial LAB with in vitro probiotic properties have been isolated from other sources, like infant and pig faeces and fermented vegetables, and used as starter cultures in dry fermented sausages resulting in high detectable populations in the final products (>6.0 log CFU/g) highlighting a potential functional value of such products (9,10,11). The isolation of LAB of meat product origin remains an issue of ongoing research since they are regarded more competitive and well-adjusted to the microenvironment of fermented sausages (12, 13). The use of such LAB isolates as starter cultures with potential probiotic superiority compared to their commercially available counterparts could improve and optimize the sausage fermentation process resulting in products with exceptional quality (13). LAB isolates with in vitro functional properties have been isolated from spontaneously fermented meat products for potential use as starter cultures in fermented sausages production (12, 14). In particular, Ruiz-Moyano et al. (11) have produced dry fermented sausages using a meat-origin Pediococcus acidilactici isolate with in
vitro probiotic properties without reporting any notable modifications on the physicochemical and sensory quality of the final products.

This study aimed to produce dry fermented sausages by combining a novel replacement of added pork fat with extra virgin olive oil and the addition of indigenous starter cultures isolated from traditional meat products. For this purpose, an edible pork fat substitute with maximum extra virgin olive oil and minimum possible water content was produced in order to totally replace the added pork fat in dry fermented sausages. Another aim was to isolate, classify and examine the most important probiotic and safety characteristics of autochthonous LAB isolates from spontaneously fermented sausages in order to evaluate their utilization as starters in fermented sausages production. The olive oil-based formulation and selected autochthonous LAB isolates were used for the production of dry fermented sausages that were evaluated for sensory, microbiological and physicochemical parameters.

MATERIALS AND METHODS

Production of edible pork fat substitute

In order to replace the added pork fat in dry fermented sausages with a substitute based on extra virgin olive oil, it was critical to ensure that the latter would be solid and stable at both 4 °C and room temperature and resemble pork fat in terms of hardness, colour and appearance. Therefore, olive oil was solidified in a formulation by utilizing turkey lean meat as an emulsifier. Turkey breast meat was selected as a source of natural proteins that would provide to the fat replacer a light colour, the anticipated technological characteristics, such as solidification, and concurrently contribute to the desired decrease of the fat content in the final products. The extra virgin olive oil formulations were heat-treated (65 °C/40 min) prior to use in order to achieve the denaturation of proteins and subsequent desired solidification of the formulation.

Different formulations were produced and evaluated during a preliminary research (data not shown). The formulation (in 100 g) with the best appearance and hardness consisted of 41.0 g turkey meat (4 °C), 16.0 g ice water, 2.5 g salt, 0.5 g phosphates (Sigma Aldrich, St. Quentin Fallavier, France) and 0.2 g white pepper. All ingredients were chopped (Kilia 30L cutter, Kilia Fleischinenfabric, Kiel, Germany) until the temperature of the mixture reached the value of 2 °C. Then, olive oil was added continuously for thorough integration and up to 35.0 g/100 g. The cutting process was completed after the addition of 4.7 g/100 g potato starch (Sigma Aldrich). The final mixture (temperature 12-14 °C) was stuffed into 40 mm diameter polyethylene-polyamide casings (RS 3000 Baby vacuum stuffer, Risco Breveti, Zanevi, Italy)
and subsequently solidified with pasteurization at 65 °C for 40 minutes. The stable formulations (stored at -18 °C) were added to the sausage mixture at the beginning of the cutting process.

Isolation and identification of indigenous LAB from meat products

Indigenous LAB present as autochthonous microbiota without any addition of starter cultures in Greek traditional dry fermented sausages (N=16) were isolated according to the ISO 15214:1998 method (15). In brief, 20 g of aseptically sliced sausage were homogenised with 180 mL of peptone water (PW, LAB M, Lancashire, UK) in a Stomacher® 400 laboratory blender (Seward Medical, London, UK) followed by ten-fold serial dilutions, pour plate inoculation of de Man, Rogosa and Sharpe agar (MRS Agar, LAB M, Lancashire, UK) and incubation at 30 °C for 72 hours. In total, 32 out of 160 randomly selected isolates from the MRSA plates were initially characterized as LAB based on positive Gram stain (Sigma Aldrich) and negative catalase and oxidase (Sigma Aldrich) activity. A method previously described by Lazou et al. (16) was used for the extraction of genomic DNA of these 32 LAB isolates to proceed to their molecular identification. In brief, one loop of cells of each isolate was mixed with 100 μL of “Lysis buffer I” (50 mM Tris-HCl, 50 mM EDTA, 4 M Guanidinium hydrochloride-GuHCl, 10 mM CaCl₂, 1% Triton X-100, and 2% N-lauroyl-sarcosine, pH 7.5; Merck, Darmstadt, Germany) and 25 μL of proteinase K (0.56 mg; New England Biolabs, Ipswich, MA, USA), and mixtures were incubated at 56 °C for one hour. Then, 250 μL of “Lysis buffer II” (50 mM Tris-HCl, 25 mM EDTA, 8 M GuHCl, 3% Triton X-100, and 3% N-lauroyl-sarcosine, pH 6.3; Merck) were added and mixtures were incubated at 70°C for 10 min. Absolute ethanol (250 μL; Merck) was added to the lysates, and each mixture was passed through a silica column (FT-2.0; G. KiskerGbr, Steinfurt, Germany) by centrifugation (8,000 × g). Columns were washed three times, once with “Wash buffer I” (25 mM Tris-HCl, 4 M GuHCl, and 50% ethanol, pH 6.6; Merck), and then twice with “Wash buffer II” (10 mM Tris-HCl, 0.1 M NaCl, and 80% ethanol, pH 6.6; Merck), followed by elution in 100 μL TE buffer (Merck).

For the molecular identification of indigenous LAB isolates, a previously described PCR assay targeting the 16s-23s rRNA intergenic spacer region was applied (17). Briefly, the 16S-23S intergenic spacer region (ISR) from these isolates was amplified using primers that annealed to conserved regions of the 16S and 23S genes. These primers were 16S/p2 (5′-CTTGTAACACCGGCACGTC-3′) and 23S/p10 (5′-CCTTTCCCTACGG-TACTG-3′), which anneal to positions 1392 to 1410 of the 16S rRNA gene and to positions 713 to 731 of the 23S rRNA gene (Lactobacillus salivarius, GenBank accession number CP002034), respectively.
The obtained PCR product corresponded to the complete 16S-23S ribosomal ISR and parts of the flanking rDNAs. PCR mixtures contained 2 μL of purified DNA, 2 μL of 10x polymerase buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 100 μM of each deoxynucleoside triphosphate, 400 μL of each primer, 200 μL MgCl₂ (Life technologies, Invitrogen, The Netherlands), 1.5 U of Expand High Fidelity PCR System (Boehringer Mannheim) DNA polymerase and nuclease-free water up to a total volume of 20 μL per reaction. DNA amplification began with a pre-incubation step at 94 °C for 7 min, followed by 40 cycles of the following thermal cycling conditions: 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s. The final extension step included 3 min incubation at 72 °C. The amplification products were electrophoresed in a 1.5% agarose gel containing 0.5% ethidium bromide and visualized by UV illumination. Subsequently, the smallest spacer region PCR product (approximately 800-900 bp) of each isolate was excised from the agarose gel and extracted using a NucleoSpin Extract II kit (Macherey-Nagel, Duren, Germany). All strands were sequenced using the PCR primers 16S/p2 and 23S/p10 and internal primers 16S/p4 (5’-GCTGGATCACCTTCTTTCT-3’) and 23S/p7 (5’-TGCAGGTACTTAGATGTTTCAGTT-3’). Sequencing was performed by utilizing a BigDye Terminator v3.0 ready reaction cycler sequencer kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer’s instructions. The obtained small intergenic spacer region sequences were compared to sequences from type cultures and other Lactobacillus strains held in GenBank (18) using the BLAST algorithm (19).

**Probiotic properties and safety characteristics**

Each of the following procedures for assessing some of the basic probiotic properties and safety characteristics of the identified LAB isolates was conducted in triplicates.

**Acid resistance and bile tolerance**

Acid resistance and bile tolerance of the isolated LAB (N=32) were examined in modified media adjusted to obtain a final pH of 2.0 and 3.0 or containing 0.3% (w/v) oxgall (Oxoid, Hampshire, UK), respectively, according to the method described by Gu et al. (20).

**Biogenic amine production**

The undesired potential tyramine (TY) and histamine (HI) forming capacity of the 32 LAB isolates were determined by high performance liquid chromatography with a gradient elution system (HPLC system, Shimadzu Corporation, Kyoto, Japan) as described by Hernandez-Jover et al. (21). Shortly, the HPLC system consisted of a system controller pump,
an autosampler, a RDM postcolumn reaction equipment and a 470 spectro-fluorometric detector. A coil of 200 cm long and 0.01 in. stainless steel tubing was used to connect the T with the detector. The separation was performed on a C18 column, 3.9150 mm, 4 μm particle size, with a matching guard cartridge.

Antibiotic susceptibility

The disk-diffusion method according to the Clinical and Laboratory Standards Institute guidelines (22) was applied to assess the antibiotic susceptibility profile of eight LAB isolates, which were selected due to their resistance in low pH values and bile salts and the inability to produce biogenic amines, towards the following 18 antimicrobial agents (concentration per disk; BD BBL™ Sensi-Disc™, NJ, USA): amoxicillin (25 mg), ampicillin (10 mg), cefotaxime (30 mg), cephalothin (30 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), clindamycin (2 mg), erythromycin (15 mg), gentamycin (10 mg), kanamycin (30 mg), nalidixic acid (30 mg), neomycin (30 mg), penicillin (10 U), streptomycin (10 mg), sulfamethoxazole + trimethoprim (23.75/1.25 mg), tetracycline (30 mg) and vancomycin (30 mg) (20,23).

Antimicrobial activity against pathogens

Based on the results of the aforementioned analyses, the antimicrobial activity of five (N=5) selected LAB isolates, was tested against the following pathogenic strains: Staphylococcus aureus F137, Staphylococcus aureus F264, Escherichia coli ATCC 11303, Salmonella Typhimurium ATCC 14028, Listeria monocytogenes ATCC 7644 and Bacillus cereus ATCC 14579. These strains were grown overnight in Tryptone Soy Yeast Extract Broth (Sigma Aldrich) at 37 ºC and a diluted suspension containing approximately 6.0 log CFU/mL was spread onto a Tryptone Soy Yeast Extract Agar (Sigma Aldrich) plate. From overnight cultures of 6.0 log CFU/mL of the tested LAB isolates, a volume of 500 μL was added to sterile paper disks (13 mm diameter), left to dry and then placed onto the previously inoculated plates that were incubated aerobically at 37 ºC for 24 h. Subsequently, inhibition zone diameters were measured with a precision calliper (Traceable™ Digital Caliper, Fisher Scientific, UK). LAB isolates with inhibition zones of 1.30, 1.30-1.80 and >1.80 cm were classified as isolates with absent (-), strong (+++) and very strong (++++) inhibition, respectively (23).

Production of dry fermented sausages with olive oil and LAB with probiotic properties

All dry fermented sausages were produced by total replacement of added pork fat using the novel extra virgin olive oil-based formulation. The lean pork and beef meat were
trimmed from visible fat and connective tissue. The recipe (per 100 g) consisted of 48.0 g lean pork meat (max 5.0 g fat), 22.0 g lean beef meat (max 7.0 g fat), 25.0 g pork fat substitute, 2.1 g curing salt (2.085 g NaCl and 0.015 g NaNO₂), 2.0 g citrus dietary fibre (FiberStar Citri-fi, River Falls, WI, United States), 0.8 g dextrose and 0.1 g white pepper. The frozen pork meat (<-18 ºC) and the extra virgin olive oil formulation (<-18 ºC) were chopped at low speed followed by the addition of the minced beef, the additives and finally the salt with the nitrites. The final mixture was chopped until the desired particle size (3-5 mm) was attained and subsequently stuffed into 40.0 mm diameter permeable cellulose casings (RS 3000 Baby vacuum stuffer, Risco Breveti, Zanevi, Italy).

One commercial probiotic culture (Lactobacillus acidophilus Alce LMGP 21381 - LA) and three indigenous LAB isolates isolated during this study that combined the most desirable probiotic properties (Lactobacillus casei 62 - LC, Lactobacillus sakei 65 - LS, Pediococcus pentosaceus 156 - PP) were selected in order to be used as starter cultures for the production of equal number of fermented sausages during three independent experiments. Sausages produced in each experiment without the addition of a LAB culture served as the control (C) treatment.

Fermentation and ripening took place in a controlled-climate unit for 21 days in total. During the first six days (fermentation period) the temperature was gradually reduced from 22 ºC to 15-16 ºC, the relative humidity from 95.0 % to 85.0 % and the air velocity from 0.7 to 0.5 m/s. Throughout the following days (ripening period), the temperature was set at 15 ºC, the relative humidity at 82.0-84.0 % and the air velocity at 0.05-0.10 m/s. Sausages produced by each treatment were sampled in duplicates for physicochemical (1st, 3rd, 7th, 14th and 21st day of production), chemical (1st and 21st day of production) and microbiological analyses (1st, 3rd, 7th, 14th, 21st day of production). Sensory evaluation of all samples was performed on the 21st day of production. Lactic acid bacteria counts were also enumerated after 4 and 6 months of storage at 4 ºC.

**Physicochemical analyses**

Weight loss ratios (PCB 1000-2, Kern & Sohn GmbH, Balingen Germany), pH values (WTW microprocessor pH-meter, WTW GmbH, Weinheim, Germany) and water activity (AQUA LAB Mod. CX-2, Decagon Devices Inc., Pullman, WA, United States) of produced sausages were measured (24).

**Chemical analyses**
Moisture was determined by drying at (102±2) °C in a drying oven overnight (Heratherm™ General Protocol Ovens, Thermo Fisher Scientific, Massachusetts, USA) to a constant weight, according to AOAC method 950.46 (24). Crude fat content was determined by extracting the fat from the dried sample using petroleum ether (Sigma Aldrich) with a Soxtherm 2000 device (S306 AK model, Gerhardt, Königswinter, Germany) (AOAC method 991.36) (21). Total nitrogen was determined in a Kjeldahltherm device (KB Digestion Systems model, Gerhardt) according to AOAC method 928.08 (24). The sample was digested with concentrated sulphuric acid (Sigma Aldrich). The nitrogen was broken down to ammonium sulphate. Caustic soda (Sigma Aldrich) was added in excess, ammonia was released by water steam distillation and trapped in a solution of boric acid (Sigma Aldrich). Titration against an acid solution was done and the nitrogen content was calculated. Ash content of the samples was determined in an amount of dried, defatted sample. Ashing was carried out at 525 °C overnight (M110 Muffle Furnaces, Thermo Fisher Scientific, Massachusetts, USA). All chemical analyses for the evaluation of dietary value were performed in duplicates.

Lipid oxidation was determined by a selective third-order derivative spectrophotometric method (25). A rapid method for measuring malondialdehyde as a marker of lipid peroxidation in sausage samples was used. Samples were homogenized with trichloroacetic acid (Sigma Aldrich), hexane (Sigma Aldrich) and butylated hydroxytoluene (Sigma Aldrich) and then centrifuged. Malondialdehyde was quantified based on the third-derivative absorption spectrum of the pink complex formed, after reaction with thiobarbituric acid reagent (Sigma Aldrich).

Microbiological analyses

The following microbiological parameters were enumerated in the produced fermented sausages using ten-fold serial dilutions, as described previously, followed by inoculation and incubation of the selective media as indicated in the corresponding ISO methods: LAB (15), coagulase positive staphylococci (26), Enterobacteriaceae (27) and yeasts-moulds (28, 29). Detection of Listeria monocytogenes (L. monocytogenes) was performed on the 1st and 21st day of production (30). A previously described multiplex polymerase chain reaction (PCR) assay (31) was used to molecularly identify five randomly-selected presumptive L. monocytogenes colonies originally grown on Agar Listeria Ottaviani Agosti (ALOA, Biolife, Milan, Italy) plates (32). This assay utilises a combination of genus-and-species specific primers and generates three possible band types indicative of bacterial, Listeria spp. and L. monocytogenes genomic DNA, respectively.
**Real-time PCR primers and conditions**

DNA extraction was performed (10) to all the countable (25-250) LAB colonies isolated on MRS agar plates from the produced fermented sausages at the beginning (day zero) and end of production (21st day) as well as after four and six months of storage at 4 ºC. A real-time PCR assay was applied in order to identify which percentage of the aforementioned LAB colonies was formed by the starter culture isolate used in each of the three corresponding treatments and to detect the potential existence of populations of the same species as the starter among the autochthonous microbiota of the control treatment. Since the overall acceptability of the fermented sausages was unknown at the beginning of their production, the real-time PCR assay was performed to LAB colonies originating only from treatments that received positive sensory evaluations. Therefore, LAB colonies isolated from the treatment with *P. pentosaceus* were excluded from molecular identification due to the observed sensory deficiencies during the evaluation of the final products. Primers targeting the conserved functional *tuf* gene for the elongation factor Tu (33) were designed for the specific detection of isolates belonging to the species *L. acidophilus*, *L. casei* and *L. sakei*. Each primer pair was designed after multiple alignments of available *tuf* gene sequences using the MEGA version 6 software (34) and checked for specificity using the BLAST algorithm (19). The specificity of each pair of primers was confirmed by applying respective real-time PCR assays using DNA templates from all three *Lactobacillus* species (*L. acidophilus*, *L. casei* and *L. sakei*) used (Table 1). The real-time PCR reactions and cycling conditions were performed in a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, United States). Amplification reactions were run in a total volume of 20 μL using 2 μL of DNA template. The optimal reaction conditions for real-time PCR were as follows: 3 units of HotStartTaq Plus DNA polymerase (Qiagen, Copenhagen, Denmark), 1X PCR Buffer (Qiagen), 2 mmol/L MgCl₂ (Qiagen), 0.2 μmol/L of each primer (Qiagen), 0.2 mmol/L dNTPs mix (Qiagen), 1X DNA-specific fluorescent dye EvaGreen™ (Biotium, Hayward, CA, United States). Cycling conditions included a preliminary denaturation step at 94 ºC for 15 min, followed by 45 cycles of 95 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 10 s. Succeeding amplification, a melting curve analysis was performed to confirm the correct amplification product by its specific melting temperature at (70-92±0.2) ºC/5 s. All samples were run in triplicates and Ct values <38 were regarded as positive results.

[Table 1]
Sensory evaluation

A trained panel of individuals working in the academia and in the food industry (N=15) evaluated the overall acceptability of dry fermented sausages according to a 9-point hedonic scale (9 = like extremely, 1 = dislike extremely) (35). The sensory panel consisted of seven trained female panelists aged between 35 and 60 and eight male panelists aged between 40 and 67. All panelists had more than two years’ experience in the sensory assessment of meat and meat products. The evaluation related to the overall acceptability of dry fermented sausages based on their appearance of the cut, colour, cohesiveness, hardness, flavour, overall acceptance (36). All sausages were evaluated on the same day. Three replicates of each sample were evaluated. The sausage order was randomized and the sausages were served in the same order to all panellists. The panellists had maximum two bites of each sample. Between samples the panellists were asked to cleanse the palate by drinking water and eat a piece of bread.

Textural evaluation

An Instron Universal Testing Machine model 1140 (Instron Ltd., Wycombe, UK) was used for the textural measurements. It was equipped with a load cell of 0–500 and 0–50 Kg for texture profile analysis (TPA) and the cut tests, respectively. The crosshead speed was set to 80 mm/min for both tests. Cylindrical samples of 23 mm in diameter and 21.5 mm in height were generated. The sausages samples were equilibrated for about an hour at room temperature (20±1) ºC before testing and their textural properties were evaluated by the method of TPA. The samples were uniaxially compressed at room temperature to 80% of their original height and each one was subjected to two subsequent cycles (bites) of compression–decompression. Hardness 1 (H1) and 2 (H2), work done on the sample during the first bite (A1) and on the second bite (A2), cohesiveness (A2/A1), springiness (S2), gumminess (G) and chewiness (K) were calculated from the obtained profiles using the MathCAD software (37). TPA test was conducted in triplicate for each of the three replications (38). The cut test was also applied to the samples using an applicable to the head of Instron tool (knife) that cut the samples in two pieces. The properties determined from the obtained curves were the force of cut (F) (the first peak of the curve indicating that the sample starts to cut) and the work done on the sample until the begging of cutting (A) (38).

Colour measurement
Colour measurements were carried out with a Hunter Lab, model Labscan 5000, spectrocolorimeter (Hunter Associates Laboratory, Inc., Reston, VA, USA) using a 10 mm port size, illuminent D65 and a 10-standard observer. CIELAB $L^*$, $a^*$ and $b^*$ values were determined as indicators of lightness, redness and yellowness. Five measurements were made on the cross-section of three (5 cm long) pieces of sausage (39).

Statistical analysis

A General Linear Model was applied to evaluate the statistical significance ($p<0.05$) of the differences observed between the starter LAB cultures regarding all the tested characteristics of the final products using the Minitab 17 Statistical Software (40). Tukey test was applied to compare the average scores that were statistically different at a confidence level of 5.0 % (40). Moreover, PanelCheck (version 1.4.0, Nofima) was used for a two-way analysis of variance for the results of sensory evaluation by panellists (41).

RESULTS AND DISCUSSION

Isolation of LAB from meat products

In the present study, the comparison of the sequenced 16s-23s rRNA gene boundary regions (GenBank accession numbers: MT431413, MT431419) obtained from the LAB isolates with those held in the GenBank database enabled the identification of Lactobacillus sakei ($N=14$), Lactobacillus casei ($N=4$), Lactobacillus plantarum ($N=2$), Lactobacillus brevis ($N=2$), Lactobacillus acidilactici ($N=2$) and Pediococcus pentosaceus ($N=8$). These LAB were isolated from traditional Lefkadas sausages and Suzuk type dry fermented sausages, which were originally chosen because they are produced by LAB adapted to their microenvironment without the addition of any commercial starter cultures. LAB that have been isolated from fermented sausages with different production technologies and without the addition of starter culture usually belong to the genera Lactobacillus, Weissella, Leuconostoc, Pediococcus, Enterococcus and Lactococcus (42). Among them, lactobacilli are most frequently isolated and, in particular, the species Lactobacillus sakei, Lactobacillus curvatus and Lactobacillus plantarum (42). Our results indicate that LAB were successfully isolated from both Suzuk type and Lefkadas sausages despite their low pH values (<4.6) and high content of spices that usually inhibit LAB growth.

Probiotic properties and safety characteristics of the LAB isolates
Acid and bile salt resistance are regarded two of the most important probiotic properties (43). Isolates of Lactobacillus casei (N=2), L. sakei (N=4) and P. pentosaceus (N=2) were able to grow in low pH values (<3.0) along with the presence of bile salts contrary to the rest of the tested LAB isolates (N=24) that were unable to grow under these conditions.

None of the isolates tested (N=32) produced histamine, whereas one isolate of L. plantarum, P. acidilactici and L. brevis produced tyramine (1755, 1577 and 1581 μg/mL of broth, respectively). Tyramine and histamine are biologically active biogenic amines produced by the microbial decarboxylation of tyrosine and histidine, respectively. Their excessive consumption can cause nervous, gastric and intestinal or blood pressure problems and, consequently, increased attention is focused on their occurrence in foods (44). Presence of precursors (free amino acids), microorganisms with amino acid decarboxylase activity and favourable conditions for growth and decarboxylation are prerequisites for the production of biogenic amines. The extensive proteolysis during ripening in dry fermented sausages can provide such precursors for subsequent decarboxylation by microorganisms. The latter are either introduced in dry sausages as starter cultures or are part of the autochthonous microbiota. Indeed, Bover-Cid & Holzapfel (45) have reported the isolation of various LAB from fermented sausages with amino acid decarboxylating activity including several L. curvatus, L. casei and L. plantarum isolates. In this study, all isolates of Lactobacillus casei, L. sakei and P. pentosaceus did not produce biogenic amines and thus were deemed safe to be used in fermented sausage production.

Lactic acid bacteria have a long history of traditional use in fermented foods technology. However, when harbouring transferable antibiotic resistance genes, they may serve as effective vehicles for antibiotic resistance transmission to other LAB or human pathogens, thus raising significant public health concerns. In recent years, increased focus has been given to foodstuffs as vehicles of antibiotic resistance genes (46, 47). To prevent the undesirable transfer of resistance to endogenous bacteria, probiotics should carry the necessary resistance in terms of surviving in the presence of non-critically important antibiotics in the human intestinal microenvironment (13). Although special purpose probiotics for use in combination with antibiotics have been developed through the introduction of multiple resistance genes to their bacterial cells, probiotics generally should not carry more resistance than it is required for a specific purpose (47). Nevertheless, LAB with susceptibility to all antibiotic categories have not been isolated as yet (48). In this study, only eight LAB isolates were able to grow in low pH values and in the presence of bile salts, and did not produce biogenic amines and, therefore, were selected to be further tested for their antibiotic resistance.
against 18 antibiotics. These isolates were found to be resistant to ampicillin, kanamycin, vancomycin and sulfamethoxazole and susceptible to streptomycin, gentamycin, tetracycline, chloramphenicol, cephalothin, clindamycin, erythromycin and cefotaxime. These findings are in accordance with results of Clementi et al. (49). Only five out of these eight isolates were selected for further investigation, based on their susceptibility to ciprofloxacin and nalidixic acid and due to the fact that the top three critically important categories of antimicrobials for public health are macrolides, 3rd-4th generation cephalosporins and quinolones (48). More precisely, L. casei (N=1), L. sakei (N=3), and P. pentosaceus (N=1) isolates exhibited resistance to less antibiotics than the rest of the LAB isolates examined and, therefore, were further selected to test their antimicrobial activity against six food-related pathogens.

Finally, only the L. sakei isolates displayed antimicrobial activity against all pathogens tested. L. casei exhibited antimicrobial activity against S. Typhimurium and B. cereus whereas P. pentosaceus showed moderate antimicrobial activity against all the pathogen isolates examined. Therefore, L. casei (N=1), L. sakei (N=1) and P. pentosaceus (N=1), which were isolated, molecularly identified and evaluated for their in vitro probiotic properties in this study, displayed desirable characteristics and were finally selected to be utilized as starters for the production of fermented sausages.

**Production of dry fermented sausages with olive oil and LAB with probiotic properties**

**Fat substitute production**

The aim of this study was to produce ‘healthier’ fermented sausages. Pork fat was totally replaced by a substitute of extra virgin olive oil and turkey proteins. Extra virgin olive oil was chosen because of its exceptional dietary value, but it had to be solidified in order to avoid technological issues. Moreover, this substitute had to be of light colour in order to replace the pork fat and the final product to have an accepted appearance for the consumers. By emulsifying olive oil with turkey proteins, the above aims were fulfilled (Fig. S1).

The olive oil-based formulation developed in this study replaced successfully the traditionally added pork fat in dry fermented sausages. The pasteurization of the formulation effectively stabilized the extra virgin olive oil in the mass of the denaturized turkey proteins. Consequently, defects such as irregular weight loss, surface wrinkling or case hardening were not observed in the final products of this study in contrast to previously reported results regarding efforts of reducing or replacing added pork fat (2, 6, 7). Sausages were produced according to the traditional technologies with pork and bovine meat and fermented for 21 days, during which the weight loss was up to 30 %. Post fermentation, sausages were pathogens...
free and ready for consumption. During sensory evaluation, they obtained high scores in all sensory properties, such as flavour, firmness, appearance, and overall acceptance. The emulsion of olive oil, forming a mosaic pattern, was visible in the sliced product in the same way that fat is visible in the conventional fermented sausages (Fig. S2).

Evaluation of physicochemical parameters

Weight loss was approximately 30 g/100 g at the 21st day of production regarding all treatments. The addition of LAB cultures did not affect significantly the weight loss of sausages during the production in comparison with the control (p=0.497-0.856). However, after the 14th day of production, LAB and control treatments exhibited lower weight loss than the rest of the inoculated treatments. There were no statistically significant differences in pH values (p=0.168-0.848) and water activity (p=0.389-0.832) throughout the production process among the different treatments and both parameters decreased sufficiently, thus, ensuring the microbiological stability and safety of the final products. All four LAB cultures tested proved successful candidates for use as starter cultures, as they fermented the available sugars and produced lactic acid, causing the reduction of the pH value. They ensured the evolution of the fermentation and, therefore, highlighted their prospective application in large-scale sausage production (43, 50, 51).

Evaluation of chemical parameters

The moisture content decreased significantly in all treatments (p<0.001) from the beginning to the 21st day of production leading to the significant (p<0.001) increase in protein, fat and ash content (Table 2). Nonetheless, the addition of the starter culture did not influence significantly, among the different treatments, the moisture (p=0.297), ash (p=0.233), fat (p=0.654) and protein (p=0.223) content of the final products. It was logical to assume that the percentage (g/100 g) of monounsaturated fatty acids increased and of saturated fatty acids and cholesterol decreased in comparison to conventional fermented sausages since the olive oil-based formulation totally replaced the traditionally added pork fat and, therefore, no relevant measurements were carried out. The presence of extra virgin olive oil instead of pork fat enhances the beneficial effects of the sausages produced in this study and is in accordance to the principles of the Mediterranean diet that promotes the consumption of unsaturated fatty acids (52). The addition of the LAB cultures did not affect significantly the oxidation of fatty acids during the fermentation and ripening up to the 21st day of the production process (p=0.501-0.663) among all treatments (Fig. 1). However, a statistically significant increase of
MDA values in all treatments was observed (p<0.001) but without the detection of any oxidation taste during the sensory evaluation of the final products.

In this study, the total replacement of added pork fat with the extra virgin olive oil formulation led to the reduction of fat in the produced fermented sausages (14.5-14.8 g/100 g fat content). This fat reduction was more than 30.0 g/100g compared to the conventional dry fermented sausages, which ranges between 30.0 and 50.0 g/100 g depending on the country or region of origin (2). This fact could allow the use of the term “low fat” to characterize the fermented sausages produced in this study according to the European Regulation 1924/2006 on health claims (53). In the case of FDA, the term “low fat” has to be used in the statement of identity of a food that bears a relative claim, followed immediately by the name of the nutrient whose content has been altered and a comparative statement (54), so “low fat, 30 % reduced fat content” could be used.

[Table 2]
[Fig. 1]

Evaluation of microbiological parameters

The process of fermentation – ripening was completed within 21 days regarding all treatments. The control samples exhibited significantly lower counts of LAB than the other treatments that included the addition of LAB cultures, though no significant differences were observed among all treatments. The counts of the added LAB cultures were approximately 7.0 log CFU/g (Fig. 2) at the beginning of the dry sausages production. The LAB population increased up to the 3rd day, remained stable until the 14th day and displayed a slight decrease on the 21st day of ripening. This reduction, although significant, did not exceed the count of one logarithm. Overall, the LAB populations ranged between 7.5-8.5 log CFU/g at the end of the production process regarding all treatments. The fact that the counts of LAB of the controls were similar to those of the other treatments is attributed to the indigenous microbial population of raw materials (meat) consisting of different LAB species and is in accordance with other studies (43, 50). These populations remained stable (ranged between 7.5-8.0 log CFU/g) and without significant decrease on the 4th and 6th month of storage (p=0.334-0.531). One of the basic aims of this study was the identification of indigenous LAB cultures with some probiotic properties that would not only be used as starters but also could survive in the final product. According to Ruiz-Moyano et al. (11), it is critical to ensure the presence of the inoculated culture in the final product in concentrations more than 6.0 log CFU/g to demonstrate that this culture competes not only with pathogens but with the indigenous
microbiota as well, survives in the final product and then is considered to have a potential functional value for the consumer. In this study, at the beginning of the fermented sausages production (day zero), only *L. sakei* was identified in low counts (3.1 log CFU/g) in the control, LA and LC treatments as part of the indigenous microbiota of the meat, according to the results of the microbiological analysis and the identification of the LAB colonies by real-time PCR. As expected, *L. sakei* population in LS treatment on the day of production was high (6.9 log CFU/g). *L. casei* and *L. acidophilus* were identified only in the treatments in which they were added (LC: 7.6 log CFU/g of *L. casei* and LA: 7.0 log CFU/g of *L. acidophilus* respectively) on the day of production. The final LAB counts identified on the 21st day of production by real-time PCR were 5.9 log CFU/g *L. acidophilus*, 7.0 log CFU/g *L. casei* and 5.7 log CFU/g *L. sakei* in LA, LC and LS treatments, respectively. The final LAB counts identified after 4 months of storage by real-time PCR were 5.7 log CFU/g *L. acidophilus*, 7.1 log CFU/g *L. casei* and 5.6 log CFU/g *L. sakei* in LA, LC and LS treatments, respectively. Only after six months of storage, the identified LAB by real-time PCR were decreased significantly to 5.1 log CFU/g *L. acidophilus*, 6.7 log CFU/g *L. casei* and 5.0 log CFU/g *L. sakei* in LA, LC and LS treatments, respectively. *P. pentosaceus* was not molecularly identified in the final products since sausages produced with this isolate obtained low scores in sensory evaluation, as it is described below, and were rejected from further evaluation. Interestingly, *L. casei* 62, isolated from traditional fermented sausages, survived in the highest counts in the final products even after six months of storage, thus, exhibiting better adaptation and competitiveness in the microenvironment of the fermented sausage compared to the commercial probiotic culture used. Therefore, *L. casei* 62 could be used in large scale production, as it prevailed against other LAB of indigenous microbiota probably due to its meat-derived origin and survived in higher counts compared to the commercial starter isolated from milk products. This fact indicates that the isolation and evaluation of autochthonous LAB of meat intended to be used as starters in the production of fermented meat products is quite promising. The need of a variety of starter cultures with probiotic properties is very important as they could provide the meat industry with products with special flavours and organoleptic characteristics in addition to a potential functional value. Various LAB isolates with *in vitro* probiotic properties have been previously isolated from different sources such as fermented milk and meat (9, 10, 11). Some of them could be promising candidates for further investigation such as the *L. casei* 62 isolated in the present study. Nevertheless, the evaluation of the *in vitro* probiotic properties of such LAB isolates should be combined with their practical use in fermented sausages production in order to evaluate their technological characteristics as well.
Consumers do not prefer dry fermented sausages for their functional value, as they tend to do for dairy products. In this study, a novel and premium meat product was produced with extra virgin olive oil and LAB that exhibited some probiotic properties in vitro. On the other hand, any reference to “probiotic properties” as a health claim cannot appear on any food unless results from specific clinical studies support such a function beyond any doubt (8, 53). However, the isolate L. casei 62 proved to be a promising LAB isolate since it exhibited some probiotic properties in vitro and its further investigation including clinical studies could prove beneficial in order to prove any in vivo functional value. This isolate in combination with the reduction of fat content and desirable alteration of the lipid acid profile, due to the presence of the monounsaturated fatty acids of extra virgin olive oil origin, could provide to the meat industry a new premium dry fermented meat product with nutritional benefits for the consumers.

Apart from LAB, the population of yeasts gradually decreased until the 7th day and increased afterwards. L. monocytogenes was not detected and moulds were below the limit of detection regarding all treatments. Enterobacteriaceae and coagulase-positive staphylococci counts were not affected significantly by the addition of LAB cultures (p=0.393-0.954) and did not survive in any treatment after the 7th and 14th day, respectively. In previous studies, staphylococci have been detected in the final products of fermented sausages, thus, raising food safety apprehensions (55). A decrease to non-detectable counts of both Enterobacteriaceae and staphylococci was observed in this study, an indicative fact of the high quality of raw materials, hygienic handling and the effective addition of LAB as starter cultures.

Sensory evaluation

The addition of different LAB did not affect significantly the appearance of the cut surface (p=0.912), colour (p=0.852), consistency (p=0.963) and hardness (p=0.664) among the different treatments. The taste/odour (p=0.886) which are contributed to the provoked lipolysis and proteolysis by the starter culture (56) and the overall acceptability (p=0.880) were not affected significantly between the different treatments (Fig. 3). All LAB isolates used in this study belong to species commonly used in fermented products. Nevertheless, the desirable sensory characteristics attributed to the final products depend on the isolate used in each case (57).
The olive oil-based fat substitute formed a mosaic pattern that was visible in the sliced final product and very similar in appearance to the visible fat in conventional fermented sausages. Moreover, this olive oil-based formulation replaced totally the added pork fat and not partially as in previous studies, where sausages with partial fat replacement displayed appearance or texture deficiencies compared to the conventional dry fermented sausages (2, 5, 6, 58). However, total replacement of the added pork fat by a so-called "konjac" matrix containing different seed and fish oils has been previously achieved (2), but the final products displayed undesirable sensorial parameters regarding hardness, appearance and overall acceptance in contrast to the fermented sausages produced in the present study. Therefore, the olive oil-based formulation produced in this study is regarded an ideal fat substitute for the production of fermented sausages.

Fig. 3

Evaluation of textural characteristics and colour

The textural parameters examined are presented in Table 4. Fat replacement with olive oil emulsions affected significantly (p<0.001) all textural properties except cohesiveness (p=0.350). Treatments with emulsions had lower values in springiness, gumminess, and chewiness in comparison to the control treatment. Moreover, olive oil emulsions increased textural properties as hardness, chewiness and cut test measurements. These objective measurements are in agreement with the sensory evaluation by experienced panellists as described previously. Colour is regarded as the most important sensory attribute of meat products by consumers (59). The addition of different LAB did not significantly affect the colour of the samples (p=0.330), but all treatments resulted in higher scores of lightness and redness in comparison to the control (p<0.001) (Table 5).

Table 4

Table 5

CONCLUSIONS

In the present study, a novel and high-quality dry fermented meat product was produced. This product managed to combine the total replacement of added pork fat by an extra virgin olive oil emulsion with turkey meat along with the addition of LAB cultures exhibiting some probiotic properties in vitro, isolated from the indigenous microbiota of traditional fermented meat products. The olive oil formulation generated a visible mosaic pattern in the sliced final product that simulated successfully the visible pork fat in the
conventional fermented sausages. Post fermentation and during sensory evaluation, the produced sausages obtained high scores in all tested parameters such as flavour, firmness, appearance and overall acceptance. Autochthonous LAB with in vitro probiotic properties could have a potential use in large-scale novel dry fermented sausages manufacture. Such isolates could be used as starters in an effort to standardize the production process and retain the typical organoleptic and sensory characteristics. Moreover, isolates like *L. casei* 62 that survived in high counts in the final products in this study, could increase the safety of fermented sausages by competing not only with pathogens but also with the indigenous microbiota and could exhibit a potential functional value for the consumer.

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CONFLICT OF INTEREST
None to declare.

SUPPLEMENTARY MATERIAL
All supplementary material is available at: www.ftb.com.hr.

AUTHORS’ CONTRIBUTION
Conception and design of the study was conducted by I. Ambrosiadis, N. Soultos, C. Dovas, and T. Magra. Experiments were conducted by I. Ambrosiadis and T. Magra. Laboratory analyses were performed by T. Magra, E. Papavergou, T. Lazou, and G. Dimitreli. Data collection and analysis were performed by T. Magra. Data interpretation was done by T. Magra, I. Ambrosiadis, N. Soultos, C. Dovas, E. Papavergou, and I. Apostolakos. Drafting the article was done by T. Magra. Critical revision and final approval of the article version to be
published was done by T. Magra, N. Soultos, C. Dovas, E. Papavergou, T. Lazou, I. Apostolakos, G. Dimitreli, and I. Ambrosiadis.

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Fig. 1. Histogram of mean values ± standard errors of lipid oxidation (ng MDA/g) of dry fermented sausages with extra virgin olive oil and LAB with probiotic properties on the day of production and 21st day of production procedure in three independent experiments
Fig. 2. Diagram of values ± standard errors (log CFU/g) of a) LAB, b) yeasts, c) Enterobacteriaceae and d) staphylococci of dry fermented sausages with extra virgin olive oil and LAB with probiotic properties during production in three independent experiments (C: control, LA: L. acidophilus Alce LMGP21381, LC: L. casei 62, LS: L. sakei 65, PP: P. pediococcus 156)
Fig. 3. Sample means (a) and spider plot (b) of sensory evaluation profiling (appearance of the cut, colour, cohesiveness, hardness, flavour, overall acceptance) of dry fermented sausages with extra virgin olive oil and LAB with probiotic properties at the 21st day of production in three independent experiments.

Samples 1: control, 2: *L. acidophilus* Alce LMGP21381, 3: *L. casei* 62, 4: *L. sakei* 65, 5: *P. pediococcus* 156

Table 1. Primers used for the molecular identification of LAB isolates with real-time PCR.

<table>
<thead>
<tr>
<th>Target Species</th>
<th>Sequences (5’ – 3’)</th>
<th>Name</th>
<th>Target gene</th>
<th>Product size/bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em></td>
<td>ACAAGGAAGCTCAAGACCAAAATCATG</td>
<td>LacF</td>
<td><em>tuf</em></td>
<td>238 bp</td>
</tr>
<tr>
<td></td>
<td>TCCAAACCAGTAACACTGACTTAAAGA</td>
<td>LacR</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. sakei</em></td>
<td>CTGACGACGTAAAGCTTGAAG</td>
<td>LSakF</td>
<td></td>
<td>224 bp</td>
</tr>
<tr>
<td></td>
<td>AACAGTTGTCTTAGCAATTTCTTCCT</td>
<td>LSakR</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>CCCTTGAAGCGTGACCAACA</td>
<td>LCaF</td>
<td></td>
<td>238 bp</td>
</tr>
<tr>
<td></td>
<td>ACGGTAGACTTGATAACATCTGGCT</td>
<td>LCaR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Mean values ± standard errors (w/%) of moisture, ash, fat and protein content of dry fermented sausages with extra virgin olive oil and LAB with probiotic properties at the day of production and 21st day of production in three independent experiments

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>t=0 day</th>
<th>t=21 day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/%</td>
<td>w/%</td>
</tr>
<tr>
<td></td>
<td>Moisture</td>
<td>Ash</td>
</tr>
<tr>
<td>C</td>
<td>(64.2±0.7)Aa</td>
<td>(3.6±0.1)Ab</td>
</tr>
<tr>
<td>LA</td>
<td>(65.0±0.2)Aa</td>
<td>(3.4±0.1)Ab</td>
</tr>
<tr>
<td>LC</td>
<td>(64.3±0.4)Aa</td>
<td>(3.4±0.2)Ab</td>
</tr>
<tr>
<td>LS</td>
<td>(64.8±0.6)Aa</td>
<td>(3.4±0.2)Ab</td>
</tr>
<tr>
<td>PP</td>
<td>(64.5±0.3)Aa</td>
<td>(3.5±0.1)Ab</td>
</tr>
</tbody>
</table>

A-C: Values with different capital superscripts indicate statistically significant differences (p<0.05) within different treatments on the same day of production.
a-b: Values with different lower superscripts indicate statistically significant differences (p<0.05) for the same treatment between the day of production and the 21st day of production.
Table 3. Mean values ± standard errors of *Enterobacteriaceae*, Staphylococci, LAB and Yeasts counts (N/log CFU/g) during fermentation of dry fermented sausages with extra virgin olive oil and LAB with probiotic properties

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>t/day</th>
<th>Control</th>
<th><em>L. acidophilus</em></th>
<th><em>L. casei</em></th>
<th><em>L. sakei</em></th>
<th><em>P. pentosaceus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterobacteriaceae</strong></td>
<td>1</td>
<td>(2.7±0.75)(^{Aa})</td>
<td>(2.8±0.75)(^{Aa})</td>
<td>(2.8±1.40)(^{Aa})</td>
<td>(2.7±0.64)(^{Aa})</td>
<td>(2.7±1.34)(^{Aa})</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(1.9±0.25)(^{Bb})</td>
<td>(1.7±0.21)(^{Bb})</td>
<td>(1.9±0.35)(^{Bb})</td>
<td>(1.6±0.21)(^{Bb})</td>
<td>(1.3±0.21)(^{Bb})</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
<td>N.D.(^{Cc})</td>
</tr>
<tr>
<td><strong>Staphylococci</strong></td>
<td>1</td>
<td>(4.0±0.21)(^{Aa})</td>
<td>(4.1±0.06)(^{Aa})</td>
<td>(3.8±0.01)(^{Aa})</td>
<td>(3.9±0.18)(^{Aa})</td>
<td>(4.2±0.00)(^{Aa})</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(2.7±0.2)(^{Bb})</td>
<td>(2.8±0.3)(^{Bb})</td>
<td>(2.9±0.0)(^{Bb})</td>
<td>(2.9±0.0)(^{Bb})</td>
<td>(3.0±0.2)(^{Bb})</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>(1.4±0.3)(^{Cc})</td>
<td>(1.4±0.3)(^{Cc})</td>
<td>(1.4±0.2)(^{Cc})</td>
<td>(2.0±0.2)(^{Cc})</td>
<td>(1.8±0.1)(^{Cc})</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
<td>N.D.(^{Dd})</td>
</tr>
<tr>
<td><strong>Lactic Acid Bacteria</strong></td>
<td>1</td>
<td>(4.9±1.0)(^{Aa})</td>
<td>(7.1±0.4)(^{Bb})</td>
<td>(7.7±0.2)(^{Bb})</td>
<td>(7.1±1.1)(^{Bb})</td>
<td>(7.5±0.1)(^{Bb})</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(7.6±0.7)(^{Ab})</td>
<td>(8.1±0.4)(^{Bb})</td>
<td>(8.4±0.4)(^{Bb})</td>
<td>(8.2±0.7)(^{Bb})</td>
<td>(7.7±0.3)(^{Ab})</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>(8.0±0.1)(^{Ab})</td>
<td>(8.5±0.2)(^{Bc})</td>
<td>(9.0±0.3)(^{Bc})</td>
<td>(8.7±0.5)(^{Bc})</td>
<td>(7.9±0.0)(^{Ab})</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>(8.2±0.1)(^{Ab})</td>
<td>(8.7±0.3)(^{Ac})</td>
<td>(8.8±0.6)(^{Ac})</td>
<td>(8.4±0.6)(^{Ac})</td>
<td>(8.3±0.0)(^{Ab})</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>(8.0±0.2)(^{Ab})</td>
<td>(8.4±0.1)(^{Ac})</td>
<td>(8.9±0.9)(^{Ac})</td>
<td>(8.4±0.4)(^{Ac})</td>
<td>(8.3±0.3)(^{Ab})</td>
</tr>
<tr>
<td><strong>Yeasts</strong></td>
<td>1</td>
<td>(4.4±0.5)(^{Aa})</td>
<td>(4.4±0.3)(^{Aa})</td>
<td>(4.6±0.1)(^{Ab})</td>
<td>(4.5±0.7)(^{Aa})</td>
<td>(4.8±0.0)(^{Aa})</td>
</tr>
</tbody>
</table>
N.D: Not Detected
A-C: Values with different capital superscripts indicate statistically significant differences (p<0.05) within different treatments of the final products.
a-d: Values with different lower superscripts indicate statistically significant differences (p<0.05) for the same treatment between the days of fermentation.

Table 4. Textural properties of fermented sausages produced with pasteurized olive oil emulsions

<table>
<thead>
<tr>
<th>Samples</th>
<th>Texture Profile Analysis (TPA)*</th>
<th>Cut test</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>(500.2±58.1)C</td>
<td>(2.3±0.2)B</td>
</tr>
<tr>
<td>LA</td>
<td>(752.9±28.6)B</td>
<td>(2.9±0.01)AB</td>
</tr>
<tr>
<td>LC</td>
<td>(859.9±28.1)AB</td>
<td>(3.2±0.3)A</td>
</tr>
<tr>
<td>LS</td>
<td>(898.2±66.3)AB</td>
<td>(3.2±0.3)A</td>
</tr>
<tr>
<td>PP</td>
<td>(988.7±72.9)A</td>
<td>(3.4±0.3)A</td>
</tr>
</tbody>
</table>

A-C: Values with different capital superscripts indicate statistically significant differences (p<0.05) within different treatments of the final products.
Table 5. Colour measurements of fermented sausages produced with olive oil emulsions

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L*</td>
</tr>
<tr>
<td>C</td>
<td>(45.2±1.3)B</td>
</tr>
<tr>
<td>LA</td>
<td>(46.3±2.1)A</td>
</tr>
<tr>
<td>LC</td>
<td>(46.6±1.6)A</td>
</tr>
<tr>
<td>LS</td>
<td>(46.2±1.4)A</td>
</tr>
<tr>
<td>PP</td>
<td>(46.8±1.9)A</td>
</tr>
</tbody>
</table>

L*: lightness, a*: redness, b*: yellowness.
A-C: Values with different capital superscripts indicate statistically significant differences (p<0.05) within different treatments of the final products
Fig. S1. Appearance of the extra virgin olive oil and turkey meat formulation (solidified with pasteurization at 65 °C for 40 minutes) that was used as pork fat substitute in this study

Fig. S2. Appearance of the cut and overall appearance of dry fermented sausages produced in this study with extra virgin olive oil and *L. casei* 62