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**Triacylglycerols hydrolysis and hydroxy- and epoxy-fatty acids release during
lactic fermentation of plant matrices: an extensive study showing inter- and
intra-species capabilities of lactic acid bacteria**

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Abstract

This study aims to show that lactic fermentation by selected starters can enrich plant matrices with hydroxy- and oxo fatty acids. The behavior of 31 lactic acid bacteria strains was investigated during the fermentation of Persian walnut, which was selected as a model growth substrate due to its inherent lipids content. The content of the following free fatty acids increased in the majority of the fermented walnut samples: linoleic, α -linolenic, palmitic, and oleic acids. The increase of diacylglycerols and, especially, monoacylglycerols levels in fermented walnuts confirmed that strain-specific bacterial lipolytic activities hydrolyzed triacylglycerols during walnuts fermentation. Twelve hydroxylated or epoxidized derivatives arising from oleic, linoleic, and linolenic fatty acids, in five groups of isomeric compounds, were also identified. In addition to the better-known lactobacilli, certain strains of *Weissella cibaria*, *Leuconostoc mesenteroides* and *Enterococcus faecalis* emerged for their lipolytic activities and ability to release hydroxy- and epoxy-fatty acids during walnuts fermentation.

Keywords: lipolysis; hydroxy fatty acids; epoxy-fatty acids; fermentation; lactic acid bacteria; walnuts.

Abbreviations

LAB, lactic acid bacteria; TAG, triacylglycerols; MAG, mono acyglycerol; DAG, diacylglycerols; CLA, conjugated linoleic acid; EPA, eicosapentaenoic acid; DHA, docosaheaxaenoic acid; LC-HRMS, liquid chromatography-high-resolution mass spectrometry; CFU, colony forming unit; CA, chemically acidified; WSE, water soluble extracts; LC-MS, liquid chromatography mass spectrometry; LC-HESI-MS, liquid chromatography-heated electro spray ionization-mass

spectrometry; HCD, collisional energy dissociation cell; HESI, heated electro spray ionization; IT, injection time; m/z, mass to charge ratio; XIC, eXtracted ion current; NCE, normalized collision energy; GLM, general linear model; PCA, principal component analysis; TIC, total ion current.

1. Introduction

1.1. Lipolytic activity of lactic acid bacteria

Lactic acid bacteria (LAB) are generally considered weakly lipolytic compared to other bacteria and fungi (McSweeney & Sousa, 2000). However, in fermented foods characterized by an extensive ripening time, such as cheeses and fermented meat, lipolysis by LAB and subsequent catabolism contribute to flavor development (Collins et al., 2003), and, under certain conditions, may lead to the release of bioactive fatty acid-derivatives (Ares-Yebra et al., 2019). LAB may contribute to the hydrolysis of triacylglycerols (TAG) to produce free fatty acids, glycerol, and intermediates such as monoacylglycerols (MAG) and diacylglycerols (DAG) (McSweeney & Sousa, 2000).

1.2. Hydroxy- and oxo fatty acids release by bacteria

Hydroxy- and oxo fatty acids are widely distributed in microorganisms, plants, animals, and insects (Takeuchi et al., 2016). Hydroxy fatty acids are saturated or unsaturated fatty acids with one or more -OH groups along their carbon branched chains, whereas oxo fatty acids comprise one or more oxo groups. Focusing on the microbial enzymes involved in the synthesis of these fatty acid derivatives, fatty acids-hydrating enzymes have been identified in various lactobacilli. These enzymes act on isolated carbon-carbon double bonds of the fatty acid, releasing hydroxy derivatives by reversible addition of water onto a double bond of an unsaturated fatty acid in a

regio- and stereo-specific manner (Takeuchi et al., 2016). Furthermore, hydroxy fatty acid dehydrogenases from lactobacilli generates oxo derivatives through dehydrogenation of the corresponding hydroxy fatty acids (Zhao et al., 2022). Hydroxy- and oxo fatty acids were often found in the feces of mammals, and several authors linked their synthesis to the metabolism of gut commensal bacteria, through metabolic pathways, which may represent a mechanism of detoxification for bacteria (Hira et al., 2018; Hosomi, Kiyono, & Kunisawa, 2020; Nagatake & Kunisawa, 2019). Many of these fatty acids were described as modulators of the human's health and diseases. Consequently, they are drawing an interest for potential use in pharmaceutical or food formulations, especially for the treatment of symptoms associated to the intestinal inflammatory diseases. Functional activities attributable to hydroxy- and oxo fatty acids include promotion of intestinal barrier function, induction of anti-inflammatory macrophages differentiation, stimulation of tight junctions-related genes expression, prevention of obesity-related metabolic perturbations, anti-diabetic effects, and suppression of food allergy effects (Hira et al., 2018; Hosomi et al., 2020; Schoeler & Caesar, 2019). The production of hydroxy- and oxo fatty acids has been also proposed as one of the mechanisms behind the anti-inflammatory properties of probiotic bacteria (Pujo et al., 2021).

1.3. Progress in the understanding of lipids metabolism in lactic acid bacteria

Although LAB encompass a wide and heterogenous group of microbial genera, only lactobacilli and species belonging to *Staphylococcus*, *Enterococcus*, and *Pediococcus* genera were previously associated to a specific lipolytic activity or to the conversion of fatty acids into hydroxy- or oxo-derivatives (Liang, Tang, Curtis, & Gänzle, 2020; Ndagano et al., 2011; Rocchetti et al., 2021). Furthermore, the production of hydroxy- and oxo fatty acids was previously investigated mainly

through *in vitro* studies by using pure fatty acids as substrate, or throughout the sausages fermentation (Kim & Oh, 2013; Liang et al., 2020; Rocchetti et al., 2021). Only a few preliminary studies have looked at the fermentation of plant matrices, such as sourdough and avocado fruits (Black et al., 2013; Filannino et al., 2020). In the context of plant matrices fermentation, the investigation was mostly limited to the synthesis of conjugated fatty acids (Mao et al. 2022). Because of the complexity of the plant matrices fermentation biochemistry and the involvement of a plethora of microbial players and enzymes, both endogenous and exogenous, there is the need to fill the current knowledge gaps.

1.4. Walnut as fermentation substrate

We aim to demonstrate that lactic fermentation by selected starters allow to enrich plant matrices in hydroxy- and oxo fatty acids. We focused our attention also on species whose potential in metabolizing lipids has so far been neglected. Therefore, we proposed to investigate the behavior of a diversified pattern of lactic acid bacteria strains during fermentation of Persian walnut (*Juglans regia* L.), which was selected as a model growth substrate because of its inherent lipids content (Guasch-Ferré et al., 2018). Triacylglycerols are the most abundant lipids in walnut, compared to phospholipids, sphingolipids, DAG, MAG, sterols, and free fatty acids, that are less abundant (Fregapane et al., 2019). Among walnut fatty acids, the most representative are oleic (18:1), linoleic (18:2), and α -linolenic (18:3) acids, which exert the well-known human health-promoting effects such as the reduction of cardiovascular risks and the improvement of lipid profile in blood (Guasch-Ferré et al., 2018). Linoleic acid is the main polyunsaturated fatty acid in walnut (58% of total fatty acids), followed by oleic (21%), α -linolenic (12%), and palmitic (6.7%) acids (Gangopadhyay et al., 2021). Some of these free fatty acids, such as α -linolenic acid and

linoleic acid, which are commonly known as omega-3 and omega-6, respectively, cannot be synthesized by humans, representing the precursors of beneficial eicosapentaenoic acid (EPA, n-3) and docosahexaenoic acid (DHA, n-3), highly engaged in human protective mechanisms (Saini & Keum, 2018). Lactic acid fermentation, considered as an effective biotechnology able to increase the nutritional properties of fermented food (Filannino et al., 2018), was indeed proposed as a green technology to boost the content of bioactive microbial derivatives of walnut fatty acids (Kim & Oh, 2013). Because of their peculiar lipid profile, the use of walnut as a growth model substrate will allow us to provide an exhaustive framework of lipid molecular species that can be linked with bacterial species- or strain-specific traits. Some previous studies have examined the fermentation of walnut-based matrices (Liu et al. 2022; Mao et al., 2022), but none of them focused on the release of hydroxy- and oxo fatty acids. Emerging evidence will gain industrial and functional relevance and facilitate the *ad-hoc* selection of starter cultures for both the fermentation of walnut-based products (Liu et al. 2022; Mao et al., 2022) and other lipid-rich plant matrices. Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS) was employed in the present study for the detection and characterization of lipids, including native and oxidized fatty acids and mono-/di-acylglycerols, in lipid extracts of Persian walnut subjected to fermentation by a diversified pool of LAB and in related control samples. MS analyses of fermented walnut samples were complemented by microbiological analysis, pH determination and quantification of organic acids and sugars.

2. Materials and methods

2.1. Chemicals

Cycloheximide, perchloric acid (HClO₄), acetonitrile (CH₃CN, LC-MS grade), 2-propanol ((CH₃)₂CHOH, LC-MS grade), chloroform (CHCl₃, HPLC grade), methanol (CH₃OH, LC-MS grade), ammonium acetate (CH₃CO₂NH₄) and water (LC-MS grade) were from Sigma-Aldrich (Milan, Italy). Fructose, glucose, mannitol, sucrose and lactic, acetic and citric acids were obtained Sigma-Aldrich (Milan, Italy). Oleic acid, linoleic acid, α -linolenic acid, palmitic acid, stearic acid, ricinoleic acid, arachidonic acid, cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and cis-4,7,10, 13,16,19-docosahexaenoic acid (DHA) were purchased from Sigma Aldrich (Milan, Italy).

2.2. Microorganisms and culture conditions

Thirty-one strains of lactic acid bacteria from the Culture Collection of the Department of Soil, Plant and Food Science, University of Bari Aldo Moro (Bari, Italy), and the Micro4Food laboratory of the Faculty of Science and Technology, Libera Università di Bolzano (Bolzano, Italy) were used as starters for walnut fermentation. To fully exploit the metabolic potential of the assorted group of lactic acid bacteria, the strains were chosen based on a high degree of heterogeneity both in terms of the species and the environment of origin. We also made sure to include key species involved in plant fermentation. Strains were previously isolated from plant-based products, cheese, sourdough, pollen, honeybee, or *Drosophila melanogaster* digestive tract (Table 1). Cultures were maintained as stocks in 20% (v v⁻¹) glycerol at -20°C and routinely propagated at 30 °C for 24 h in MRS broth (Oxoid, Basingstoke, Hampshire, United Kingdom) except *Streptococcus thermophilus* and *Lactococcus lactis* cultures, that were propagated at 37°C for 24 h in M17 broth (Oxoid).

2.3. Walnut fermentation

Fresh walnut (*Juglans regia* L.) were purchased from a local supermarket in Bolzano, Italy. They were shelled by hand, chopped into small pieces and blended with a Classic Blender (MB 550, Microtron, Italy). Resulting homogenate, supplemented with sterile distilled water 10% (v w⁻¹), was singly fermented by pure culture of lactic acid bacteria strains. Cells were cultivated in MRS or M17 broth until the late exponential growth phase (ca. 24 h), washed twice in 50 mM phosphate buffer, pH 7.0, and re-suspended into walnut homogenate at the initial cell density of ca. Log 7 CFU g⁻¹. After the inoculum, the fermentation was carried out at 30°C up to 48 h, leading to samples defined as Fermented-Nuts. The time of fermentation and size of inoculum were defined based on preliminary trials where growth and acidification capability of some lactic acid bacteria strains were investigated (data not shown). Walnut without microbial inoculum (Unstarted-Nuts) or chemically acidified with lactic acid (final pH of ca. 4.65) (CA-Nuts) were incubated under the same conditions, representing the two controls. Samples before fermentation/incubation (Raw-Nuts) were also considered. Before being subjected to compositional analysis, Raw-, Unstarted-, CA- and Fermented-Nuts were directly frozen, then freeze-dried using a pilot scale lyophilizer (Epsilon 2-6D LSC plus freeze-drier, Martin Christ, Osterode am Harz, Germany), and grounded.

2.4. Microbiological analysis

Ten grams of raw-, unstarted-, CA- and fermented nuts in sterile 0.9% (w v⁻¹) sodium chloride solution were homogenized with a Classic Blender (PBI International Milan, Italy) for 2 min at room temperature. Most LAB were enumerated on MRS agar, containing 0.1% of cycloheximide (Sigma Chemical Co., Milan, Italy), at 30°C for 48 - 72 h under anaerobic conditions. *Streptococcus* and *Lactococcus* species were enumerated on M17 agar, containing 0.1% of

cycloheximide (Sigma Chemical Co., Milan, Italy), at 37°C for 48 - 72 h under anaerobic conditions.

2.5. Physical and biochemical analyses

The pH was measured by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland). In order to determine the consumption of carbohydrates and the synthesis of organic acids during walnut fermentation, freeze-dried walnut powder (2 g) was extracted with 20 mL of water/perchloric acid (95:5, v:v) (Tlais et al., 2022). Mixture was sonicated (amplitude 60) using a macro-probe sonicator (Vibra-Cell sonicator; Sonic and Materials Inc., Danbury, CT) for 1 min (2 cycles, 30 s / cycle, 5 min interval between cycles) in an ice-bath. The suspension was subjected to stirring conditions at 25°C for 1 h, kept at 4°C overnight, and centrifuged for 10 min at 11,000×g. Water-soluble extracts (WSE) were filtered and stored at -20°C until further use. Concentrations of glucose, fructose and mannitol were determined through a HPLC system Ultimate 3000 (Thermo Fisher, Germering, Germany) equipped with a Spherisorb column (Waters, Millford, USA) and a Perkin Elmer 200a refractive index detector (Tlais et al., 2022). Lactic, acetic and citric acids were determined by LC-UV analysis, based on an Ultimate 3000 chromatograph (Thermo Fisher) equipped with an Aminex HPX-87H column (ion exclusion, Biorad, Hercules, CA, USA) and a UV detector operating at 210 nm (Tlais et al., 2022). Organic acid and sugar standards were purchased from Sigma-Aldrich (Milan, Italy).

2.6. Preparation of lipid standards

Nine analytical standards, purchased by Sigma Aldrich (Milan, Italy), were used to develop the LC-HRMS method to be subsequently used for the detection and quantification of fatty acids present in walnut. Most standards were typical fatty acids occurring in walnut, namely, oleic acid,

linoleic acid, α -linolenic acid, palmitic acid and stearic acid. Ricinoleic acid was also included, to be used as a model for hydroxylated fatty acids, that might be formed upon walnut fermentation. Finally, arachidonic acid, cis-5, 8, 11, 14, 17-EPA and cis-4,7,10, 13,16,19-DHA were included among standards as models of polyunsaturated fatty acids, that might eventually be present as a result of the fermentation processes. Standard stock solutions of fatty acids were prepared individually in a CH₃CN/(CH₃)₂CHOH/H₂O (65:30:5 v:v:v) mixture, with the exception of stearic and palmitic acids, that were dissolved in a CHCl₃/ CH₃OH (1:1 v:v) mixture due to the high hydrophobicity related to their saturated carbon chains. HPLC-grade solvents were used for the preparation of the standard stock solution. A combined standard spiking solution containing all analytical standards was prepared by diluting the respective stock solution in pure CH₃OH (LC-MS grade). The combined standard spiking solution was then used to prepare, by dilution, calibration solutions at decreasing concentrations. The final standard solutions were stored at -20°C.

2.7. Lipid extraction from walnuts

The procedure employed for lipid extraction was based on a modified Bligh and Dyer protocol (Bligh & Dyer, 1959), due to the dried texture of the matrix. The lipidic fraction was extracted from raw-, unstirred-, CA- and fermented-walnut after 48 h of incubation at 30°C. One gram of each freeze-dried sample was mixed with 10 mL of pure CHCl₃ (HPLC grade) and vortexed for 2 min. The sample suspensions were stirred for 15 min at 150 rpm and room temperature and then subjected to a decantation phase for 2 h at 4°C. The resulting supernatants were recovered, filtered using Whatman paper (GE healthcare UK Ltd., Little Chalfont, UK) and diluted with pure CH₃OH (1:10, v:v). Finally, the samples were subjected to a second decantation phase at 20°C overnight

and only the final supernatant was subjected to LC-HRMS analysis through a Liquid Chromatography-Heated Electro Spray Ionization- Mass Spectrometry (LC-HESI-MS) instrumental apparatus.

2.8. LC-HRMS instrumental apparatus and operating conditions

LC-HRMS analysis were performed using a LC-MS platform composed of an Ultimate 3000 RSLCnano system, coupled to a Q-Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The LC-MS system was equipped with a Higher Collisional energy Dissociation cell (HCD) and a HESI (Heated Electro Spray Ionization) interface was adopted for LC-HRMS coupling. Before LC-HRMS analysis, the mass spectrometer was periodically calibrated by direct infusion-HESI-HRMS analysis of positive and negative ion calibration solutions provided by the manufacturer. A mass accuracy better than 5 ppm was achieved. For chromatographic separation and MS detection of fatty acids, the LC-MS operating conditions reported in Losito et al. (2018) were used, with few modifications. A Supelco Ascentis Express C18 column (150 × 2.1 mm ID, 2.7 µm particle size) was used for chromatographic separations of lipid fractions, by applying the following binary gradient program: 0–50 min, linear from 80 to 100% B; 50–60 min, isocratic at 100% B; 60–65 min, return to the initial composition, followed by a 20-min equilibration time (solvent A = water + 2.5 mM ammonium acetate; solvent B = methanol + 2.5 mM ammonium acetate). The flow rate was 0.3 mL min⁻¹ and the temperature of the column was set at 31°C. MS detection following chromatographic separation was performed in Full-MS mode and in negative polarity. The main electrospray and ion optic parameters adopted during LC-MS acquisition were the following: sheath gas flow rate, 10 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); spray voltage, - 3.50 kV (negative polarity); capillary temperature,

350°C; S-lens Radio frequency level, 55 arbitrary units. The settings for the Q-ExactiveTM mass spectrometer were the following: mass scan range, 150–850 *m/z*; resolution, 75.000 (FWHM at *m/z* 200); Automatic Gain Control (AGC) Target, 1*10⁶ ions; maximum injection time (IT), 100 ms. Chromatographic peaks corresponding to walnut major fatty acids (detected as [M-H]⁻ ions in the present study) were recognized by comparing retention times with those obtained for the corresponding standards and confirmed by a search on the *LipidMaps* database, freely accessible on the Internet (<http://www.lipidmaps.org>), using as input the *m/z* value retrieved for the monoisotopic peak of each fatty acid, setting a mass tolerance of ± 0.005 *m/z* units and considering [M-H]⁻ ions. Peak areas, obtained from eXtracted Ion Current (XIC) chromatographic traces, *i.e.*, chromatograms created by extracting the ion current from HRMS spectra in a *m/z* interval including the monoisotopic peak, were used as a measurement of MS response. They were thus employed to perform calibrations based on commercial standards of palmitic, oleic, linoleic, and α-linolenic fatty acids and, afterwards, for the quantification of these fatty acids in walnut lipid extracts. The XcaliburTM v. 3.1 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for the control of the Q- Exactive plusTM spectrometer and for data elaboration.

2.9. Tentative identification of MAG and DAG in LC-HRMS spectra obtained from walnut lipid extracts

To search for other lipid classes eventually present in walnut samples, accurate *m/z* values retrieved from HRMS spectra averaged under further chromatographic peaks, *i.e.*, not corresponding to those of major fatty acids, were used as the input for a search in the *LipidMaps* database. A mass tolerance of ± 0.005 *m/z* units was set also in this case, but acetate adducts [M + CH₃COO]⁻ were

proposed as the ions potentially generated under negative polarity from further lipid compounds. As a result, several of those m/z values were found to correspond to MAG and DAG (*vide infra*).

2.10. Tentative identification of oxidized fatty acids by LC-HRMS and MS/MS analysis

Peaks detected at low retention times in LC-HRMS chromatograms and corresponding neither to major fatty acids nor to DAG/MAG were investigated for their possible correlation with oxidized fatty acids. Firstly, the m/z values corresponding to hydroxylated, epoxidized and carbonylated derivatives of fatty acids, like those previously reported in the literature (Losito et al., 2018; Fiorino et al., 2019), were used for ion current extraction from LC-HRMS data sets obtained for walnut lipid extracts. Five values, related to putative mono-hydroxy/epoxy or to di-hydroxy derivatives emerged from this preliminary search and the corresponding ions were selected as precursors for MS/MS analysis, in order to obtain further information on their chemical structure. LC-MS/MS analysis on lipid extracts was carried out in negative ionization mode, and the instrumental setting were the following: mass scan range, 150–850 m/z ; default charge state, 1; resolution, 17.500 (FWHM at m/z 200); AGC target, 1×10^5 ions; maximum injection time, 50 ms; isolation window, 1 m/z (the monoisotopic peak of each target ion was selected for subsequent fragmentation); normalized collision energy (NCE), 40 eV. The chromatographic, electrospray and ion optic parameters adopted during LC-MS/MS acquisition were the same as those reported in section 2.7. The ChemDraw Ultra 12.0.2 (Cambridge Soft Corporation, Cambridge, MA, USA) software was employed to draw chemical structures both for the precursor ions and for hypothetical product ions generated upon fragmentation.

2.11. Statistical analysis

All analyses were performed in triplicates on three biological replicates. Data were submitted to analysis of variance by the General Linear Model (GLM) of the R statistical package (R, version 1.6.2, available at the Internet address: rcompanion.org/handbook/). When significant effects were determined, pairwise comparison of treatment means was achieved by Tukey-adjusted comparison procedure at a P value of <0.05. Data representing concentrations, in the case of major fatty acids (palmitic, oleic, linoleic and α -linolenic), and areas of peaks retrieved from XIC chromatograms, in the case of oxidized fatty acids (for which no standard was available), were subjected to Principal Component (PCA) analysis after centering and scaling. Hierarchical clustering analysis, using the default method available in R and based on the Euclidean distance and the McQuitty linkage, was performed on data referring to organic acids, sugars, pH, and LAB cell density.

3. Results and discussion

3.1. Microbiological analysis

The high concentration of biogenic compounds, mainly unsaturated fatty acids, makes walnut fruit (*Juglans regia* L.) a tremendous raw matrix, which deserves to be fully investigated also following its fermentation. To the best of our knowledge, no studies have previously considered the potential of walnut as microbial substrate during lactic acid fermentation. The pathways of microbes in a metabolic labyrinth were recently integrated to drive plant fermentation (Filannino et al., 2018). Based on inherent enzyme portfolio, the aptitude of microorganisms to perform metabolic routes is species and strains dependent (Di Cagno et al., 2019). Thirty-one strains of LAB belonging to 16 species previously isolated from different sources were used as starters. Strains were chosen based on several technological and functional traits, such as the adaptation to environmental niches

rich in phenolics (Di Cagno et al., 2011; Filannino et al., 2016), the synthesis of bioactive compounds (Di Cagno et al., 2016, 2017; Tlais et al., 2021), or the capability to modify the fatty acids profiles of raw plant matrices (Di Cagno et al., 2017; Filannino et al., 2020).

Only a few microbial species, mostly fungi, are known to live inside the walnut fruit due to harsh conditions, which could be explained by the high abundance of fatty acids and polyphenols with powerful antimicrobial features (Pardatscher & Schweigkofler, 2009). As expected, lactic acid bacteria were not detected in 10 g of Raw-, Unstarted- and CA-Nuts. After 48 h of incubation, the number of lactic acid bacteria in Fermented-Nuts increased from ca. 7.0 to 7.74 ± 0.15 Log CFU g^{-1} (minimum increase) and 9.13 ± 0.39 Log CFU g^{-1} (maximum increase). *P. parvulus* S5w1, *E. faecalis* KAFEPL63 and *A. kunkeei* PL13 were the only species that showed no or negligible increase in cell density. On the other hand, the cell density of LAB in walnut fermented with *A. kunkeei* BV61 was among the highest values ($P < 0.05$) (Figure 1A and Table S1).

3.2. Analysis of pH, sugars and organic acids

The acidification capability of LAB mirrored their growth. Raw-Nuts had an initial pH of 5.84 ± 0.01 . After incubation, Unstarted-Nuts had slightly but significantly ($P < 0.05$) higher pH (5.95 ± 0.03) than Raw-Nuts. After fermentation, the lowest ($P < 0.05$) values of pH were found in samples fermented with *Lactiplantibacillus pentosus* 0253 and *Lactiplantibacillus plantarum* CB5, DC400 and AVEF17 (4.51 ± 0.01 , 4.68 ± 0.08 , 4.73 ± 0.01 and 4.79 ± 0.01 pH units, respectively), whereas samples fermented with *Lc. lactis* AFII1, *A. kunkeei* PL13, *L. paracasei* AFII5 and *E. faecalis* KAFEPL63 showed the highest values of pH (5.62 ± 0.01 - 5.74 ± 0.07 pH units). For the other strains, the values of pH ranged from 4.88 ± 0.06 to 4.53 ± 0.03 pH unit (Figure 1A and Table S1). Sucrose, fructose and glucose were the most abundant sugars in Raw-Nuts (31.13 ± 0.12 , 6.67 ± 0.31 and 4.81 ± 0.22 mg g^{-1} DM, respectively). During incubation, sucrose was

343 extensively hydrolyzed in control and fermented samples (Figure 1A and Table S1). This might
 344 be due to the microbial sucrase-type enzymes, which hydrolyzed sucrose into glucose and fructose
 345 (van Hijum et al., 2006) or to high temperature during incubation, which affects phosphoprotein
 346 activity involved in the sucrose metabolism (Qin et al., 2020). Sucrose hydrolysis was associated
 347 with the release of fructose and glucose (Figure 1A and Table S1). Glucose was found at highest
 348 concentration levels in Unstarted-Nuts ($28.19 \pm 0.44 \text{ mg g}^{-1} \text{ DM}$) and CA-Nuts ($26.65 \pm 0.77 \text{ mg}$
 349 $\text{g}^{-1} \text{ DM}$). Similarly, high levels of fructose were found in Unstarted-Nuts ($28.19 \pm 0.44 \text{ mg g}^{-1} \text{ DM}$)
 350 and CA-Nuts ($26.6 \pm 0.77 \text{ mg g}^{-1} \text{ DM}$), and walnuts fermented with *W. cibaria* strains ($20.82 \pm$
 351 0.26 and $18.27 \pm 0.77 \text{ mg g}^{-1} \text{ DM}$), and *Lc. Lactic AFII1* ($20.40 \pm 1.47 \text{ mg g}^{-1} \text{ DM}$). Other strains
 352 showed variable concentrations of glucose and fructose (Figure 1A and Table S1). The decrease
 353 of fructose reflected the accumulation of mannitol in few fermented samples. Fructose was used
 354 by *Lev. brevis* DIV15, *A. kunkeei* BV61 and by all *Leuconostoc* species as alternative external
 355 electron acceptor to produce mannitol (Yang et al., 2020). Lactic acid was the main microbial
 356 metabolite released during fermentation, followed by acetic acid. Raw-Nuts showed low
 357 concentrations of lactic, acetic and citric acids (1.72 ± 0.02 , 0.22 ± 0.01 and $0.59 \pm 0.01 \text{ mg g}^{-1}$
 358 DM , respectively). The highest level of lactic acid was found in walnut fermented with *Lacti.*
 359 *plantarum* strains ($11.38 \pm 0.01 - 19.66 \pm 0.02 \text{ mg g}^{-1} \text{ DM}$) followed by *Lacti. pentosus* strains
 360 ($10.90 \pm 0.02 - 18.19 \pm 0.02 \text{ mg g}^{-1} \text{ DM}$) and *A. kunkeei* BV61 ($12.71 \pm 0.03 \text{ mg g}^{-1} \text{ DM}$). Values
 361 of acetic and citric acids were in the range $0.14 \pm 0.01 - 2.22 \pm 0.03 \text{ mg g}^{-1} \text{ DM}$ and $0.28 \pm 0.00 -$
 362 $1.15 \pm 0.01 \text{ mg g}^{-1} \text{ DM}$, respectively. Walnut fermentation with *Levilactobacillus brevis* MD19
 363 and DIV15 led to the highest ($P < 0.05$) amounts of acetic and citric acids, respectively (Figure 1A
 364 and Table S1). Based on microbiological and biochemical characterization, samples were grouped
 365 into 5 clusters (A-E) (Figure 1B). Raw-Nuts were individually grouped in cluster C, whereas

Unstarted-Nuts and CA-Nuts were grouped in cluster D. Cluster A included *Leuc. mesenteriodes* S3d1, *Lev. brevis* DIV15 and *A. kunkeei* BV61. Cluster B included *W. cibaria* PEP23F, *F. rossiae* UNIBZ20, *L. paracesei* UNIBZ27, *S. thermophilus* UNIBZ81 and all strains of *Lacti. plantarum*, *Lacti. pentosus* and *Limosilactobacillus fermentum* species (Figure 1B). The remaining strains were grouped in cluster E. Adaptation of lactic acid bacteria strains to walnut conditions via sugar and organic acid metabolisms was partially linked to species-specific features, but several traits appeared to be strain-dependent (Di Cagno et al., 2019).

3.3. Quantitative LC-HRMS analysis of free fatty acids

Nine analytical standards were used for the identification and eventual quantification of the main fatty acids detected in fermented and untreated walnuts using LC-HRMS. Among them, only linoleic, α -linolenic, palmitic and oleic acids were markedly detected, while DHA, EPA, stearic, ricinoleic, and arachidonic acids were present in negligible amounts and, therefore excluded from quantitative determinations. The most abundant fatty acid in raw walnut was linoleic acid ($0.57 \pm 0.03 \text{ mg g}^{-1} \text{ DM}$), followed by α -linolenic ($0.31 \pm 0.01 \text{ mg g}^{-1} \text{ DM}$) and palmitic acids ($0.32 \pm 0.01 \text{ mg g}^{-1} \text{ DM}$) and, at lower concentration, oleic acid ($0.21 \pm 0.01 \text{ mg g}^{-1} \text{ DM}$) (Figure 2 and Table S2). Since linoleic acid is considered the primary fatty acid in walnut, our results mirrored what has been reported in the literature (Gangopadhyay et al., 2021). There were no significant differences in the four monitored fatty acids among Unstarted-Nuts, CA-Nuts and Raw-Nuts ($P > 0.05$), thus ruling out the role of temperature or acidification in changing the fatty acid profile. As shown in Figure 2, walnut fermented with most LAB exhibited an increase of the fatty acids content with some exceptions. Walnut fermented with *W. cibaria* PEP23F revealed the most noticeable ($P < 0.05$) increase in the final concentration of all assayed fatty acids, followed by

389 *Leuc. mesenteroides* S3d1 and *E. faecalis* KAFEPL63 (Figure 2). When fermented with *W. cibaria*
390 PEP23F, the contents of linoleic, α -linolenic, palmitic and oleic acids of walnut were 2.53 ± 0.05 ,
391 2.00 ± 0.02 , 0.97 ± 0.05 and 1.29 ± 0.04 mg g⁻¹ DM, respectively (Figure 2). The increased amounts
392 of free fatty acids can be traced to biosynthetic pathways, or more likely to esterolytic/lipolytic
393 enzymes capable of hydrolyzing TAG, DAG and MAG. Premising that LAB are not remarkable
394 for their lipolytic activity, it is known that some LAB species possess an intracellular system of
395 lipases and esterases. Although the detected activity was limited to short- and medium-chain fatty
396 acid esters, such enzymes were predominantly attributed to lactobacilli, *Lactococcus* spp. and
397 *Streptococcus* spp. (Collins et al. 2003). Nevertheless, species belonging to *Weissella*,
398 *Enterococcus*, and *Leuconostoc* genera are often isolated during spontaneous fermentation of meat,
399 fish, and dairy products (Li et al., 2020), which are rich in fats, and some of their strains were
400 previously associated with lipolytic activity (Fuka et al., 2020). Strains belonging to the same
401 species induced different changes in the substrate, as showed by the Principal Component Analysis
402 based on the concentrations found for the four main fatty acids (Figure S1). Walnut fermented
403 with *W. cibaria* PEP23F appeared as an outlier due to the highest production of all monitored fatty
404 acids. On the other hand, *W. cibaria* P9 showed much lower concentrations of monitored fatty
405 acids. Such differences can be found within most of the screened species, demonstrating that the
406 release of fatty acids is depending on the strain rather than the species, albeit with some exceptions.
407 All strains belonging to *Lacti. plantarum* showed a similar trend in palmitic acid release in
408 fermented walnut, although only *Lacti. plantarum* CB5 showed a significant ($P < 0.05$) increase
409 compared to Raw-Nuts. Palmitic acid is the only saturated fatty acid detected, and this result
410 suggests that the specific enzymes present in *Lacti. plantarum* may activate the metabolic pathway

involving saturated fatty acids. *Lim. fermentum* UNIBZ15 and F1 also showed overlapping fatty acid profiles.

3.4. Identification of TAG hydrolysis products in walnut lipid extracts by LC-HRMS

Aiming at a better understanding about the lipid metabolism by LAB during the fermentation, HRMS spectra underlying further peaks detected in LC-HRMS Total Ion Current (TIC) chromatograms of fermented walnut extracts were carefully evaluated. As reported in the literature, fatty acids are only a part of the total fat content in walnuts and other lipid classes can be found, such as TAG (Fregapane et al., 2019). To verify if fatty acids found in fermented walnut samples were also released from TAG, lipolysis by-products of the latter, namely DAG and MAG, were searched for. The LC-HRMS TIC chromatograms obtained for the apolar fraction of Raw- (panel A), Unstarted- (panel B), CA- (panel C) nuts and walnut fermented with *W. cibaria* PEP23F (panel D) are reported in Figure 3. Fermentation with *W. cibaria* PEP23F was selected as a representative condition because of the high content of free fatty acids (Figure 2 and Table S2), which suggests a boosted hydrolysis occurring on TAG. As emphasized in Figure 3, MAG and DAG species were identified in the four samples, following the procedure described in Section 2.8. Accurate m/z values enabled the retrieval of the sum compositions of those compounds (*i.e.*, the total numbers of carbon atoms and C=C bonds located on their chain/chains). As expected, DAG were eluted later than MAG from the C18 chromatographic column, due to their higher hydrophobicity. Furthermore, for the same number of carbon atoms on the side chains, the retention time decreased as the number of C=C bonds increases, due to the increase in polarity. This effect is evident in the case of DAG with 36 carbon atoms (see Figure 3).

As reported in Figure 3D, DAG and MAG were also present, together with the main fatty acids, especially the linoleic acid (18:2), in the non-fermented walnut. However, as shown in panel D, referring to the walnut fermented with *W. cibaria* PEP23F, the concentration of DAG decreased significantly with fermentation, while the concentration of fatty acids increased and the peak related to the major MAG detected (18:2) also appeared more intense. Interestingly, accurate m/z values compatible with those of the deprotonated forms ($[M-H]^-$ ions) of oxidized fatty acids, particularly hydroxy- or epoxy- fatty acids, were retrieved from spectra underlying chromatographic peaks eluting within 5 min from the chromatographic column (see the yellow area in Figure 3). Not surprisingly, these fatty acids were eluted earlier than the main fatty acids (green area in Figure 3). Indeed, the presence of oxygenated functionalities on their chains led to a drastic decrease in hydrophobicity, and, consequently, in the affinity for the C18 stationary phase.

Due to their importance in terms of lipid metabolism of LAB, a semi-quantitative investigation, based on areas of peaks detected in XIC chromatograms, was made on MAG, DAG and, after their structural characterization by MS/MS, also on oxidized fatty acids detected in fermented walnut samples.

3.5. Trend of MAG and DAG as TAG hydrolysis products in walnut lipid extracts

Hydrolytic processes on TAG might produce valuable free fatty acids, MAG, DAG, and glycerols. Monoacylglycerols and DAG represent TAG derivatives and an increase of their abundance in fermented walnuts might indicate the occurrence of TAG hydrolysis. In the present study thirteen m/z values were attributed to the acetate adducts ($[M+CH_3COO]^-$) of putative MAG (5 compounds) and DAG (8 compounds), with a mass accuracy better than 1 ppm (Table S4). The corresponding

peaks in XIC chromatograms were obtained for Raw-, Unstarted-, CA- and Fermented walnut extracts, and employed to monitor the eventual variation in the concentration of the corresponding compounds, under the assumption that the dependence of chromatographic peak area on concentration was the same for all of them. The results referred to the DAG and MAG detected in the lipid extracts are summarized in Figure 4, where chromatographic peak areas obtained for the same compound in the controls and, for the sake of example, in extracts of walnuts fermented by *W. cibaria* PEP23F are reported. A significant ($P < 0.05$) increase of the response referred to all MAG was observed in walnuts fermented with *W. cibaria* PEP23F compared to the controls, which showed almost similar intensities instead. On the other hand, no significant ($P > 0.05$) changes were found for DAG during fermentation with *W. cibaria* PEP23F, with the only exception for DAG (36:4), which showed a significant ($P < 0.05$) increase in the fermented sample compared to the controls. Based on our findings, the more obvious hypothesis is that strain-specific bacterial lipolytic activities can act on the TAG during walnuts fermentation, releasing MAG, DAG, and free fatty acids as hydrolysis products (Figure 2 and Table S2).

3.6. Identification of oxidized fatty acid derivatives by LC-MS/MS analysis

Five m/z values referred to deprotonated forms ($[M-H]^-$ ions) of putative oxidized fatty acids were obtained from HRMS spectra related to chromatographic peaks eluting at retention times lower than 5 min. They were compatible, within the 5-ppm mass accuracy typically achieved with the employed mass spectrometer, with exact m/z values 293.2122, 295.2279, 297.2435, 311.2228 and 313.2384, potentially corresponding, respectively, to hydroxylated (or epoxidized) forms of fatty acids with sum compositions 18:3, 18:2, 18:1, and to di-hydroxylated (or hydroxylated and epoxidized) forms of fatty acids with compositions 18:2 and 18:1. Notably, the hydroxylation (the

introduction of a OH group in place of a H atom linked to a C atom) and the epoxidation (the introduction of a O atom as a “bridge” between two carbon atoms previously involved in a C=C bond) of a specific fatty acid lead to compounds with the same molecular mass. In order to distinguish these compounds and, more generally, to retrieve structural information on oxidized fatty acids, MS/MS acquisitions were performed on the five precursor ions and the accurate m/z values found for their most diagnostic product ions, along with the hypothesized chemical structures, have been reported in Figure 5. As apparent, two or three isomeric species were hypothesized for four of the five m/z values related to oxidized fatty acids, with differences consisting in the position of the OH group(s) and of C=C bonds, according to the case.

The procedure followed to find the most likely chemical structures for oxidized fatty acids was quite complex. Indeed, starting from major unsaturated fatty acids detected in walnut extracts, *i.e.*, oleic, linoleic and α -linolenic ones, all possible locations of OH or epoxy functionalities on their acyl chains were considered. Afterwards, fragmentation pathways were hypothesized for each proposed structure, considering, as a guide, those previously observed for hydroxylated/epoxidated fatty acids under similar collisional energy conditions (Losito et al., 2018). As inferred from Figure 5, typical fragmentations involved C-C bond breakages, leading to ionic fragments bearing a new C=C bond or a simple saturated C atom at one end of their structure. Notably, the most common sites of chain breakage were close to carbon atoms involved in hydroxylation or epoxidation, in accordance with previous findings (Losito et al., 2018). This feature was fundamental to hypothesise the location of the OH group(s) or of the epoxy bridge, according to the case. As also evidenced in Figure 5, fragmentation pathways were further complicated by the possibility of intra-molecular charge transfer in precursor ions. Indeed, a proton was occasionally found to be detached from a OH group, or even from a carbon atom located

502 between two C=C bonds (see the product ion associated to the exact m/z 121.1023 in Figure 5),
503 and transferred towards the carboxylate group, which is the most likely site for negative charging.
504 Such processes are obviously very unlikely in solution phase, but they may become possible during
505 gas phase collisional dissociation, especially if the new position of negative charge is stabilized.
506 In the case of the carbon-based negative charge of the fragment at m/z 121.1023, stabilization is
507 likely due to resonance with the two adjacent C=C bonds.

508 It is also worth noting that, based on the type of product ions recognized, the OH group of some
509 precursor ions had to be located directly on a carbon atom previously involved in a C=C group of
510 a precursor fatty acid; an example is provided by 9-hydroxy-18:3 fatty acid, resulting from
511 hydroxylation of α -linolenic acid (see Figure 5). This type of hydroxylation is often reported as a
512 consequence of oxidation of unsaturated lipids induced by Reactive Oxygen Species (ROS), with
513 the displacement of a C=C bond along the chain and the change of its geometry from *cis* to *trans*
514 (Porter et al., 1995). In the cited example, the C=C bond originally located between C9 and C10
515 is displaced between C10 and C11.

516 Turning to specific structures reported for precursor ions in Figure 5, three isomeric compounds
517 were found to be related to ions having m/z 293.2122, a value consistent with the occurrence of
518 three C=C bonds and one OH groups on a side chain including 18 carbon atoms. After considering
519 the locations of the three C=C bonds for α - or γ -linolenic acids, different possible locations for the
520 OH group introduced upon fermentation and the structures of fragment ions obtained from MS/MS
521 analyses, it was found that two of the three isomeric compounds corresponded, respectively, to γ -
522 linolenic acid hydroxylated on C16 and α -linolenic acid hydroxylated on C17. MS/MS data
523 obtained for the third compound were consistent with the structure of 9-hydroxy-octadeca-
524 10,12,15-trienoic acid; this oxidized fatty acid was still originated by α -linolenic acid but the

525 hydroxylation occurred on C9, with displacement of the C9-C10 double bond on the C10-C11
526 bond, thus making this double bond conjugate with the C12-C13 double bond originally present
527 on the α -linolenic acid structure.

528 Three isomeric species were also hypothesized for ions compatible with an exact m/z 295.2279.
529 As emphasized in Figure 5, all were generated by linoleic acid, the main fatty acid detected in
530 walnuts, and two of them corresponded to mono-hydroxylated derivatives. In one case the OH
531 group was placed on C15, whereas in the other the OH group was linked to C13, with displacement
532 of the original C12-C13 double bond between C11 and C12, according to the process explained
533 before. Notably, recent studies attributed anti-inflammatory properties to hydroxy linoleic acid in
534 mice and mammalian cells (Paluchova et al., 2020). As evidenced in Figure 5, the third isomer
535 related to the m/z 295.2279 was the only epoxidized fatty acid for which specific evidence was
536 obtained from MS/MS analyses; in this case the epoxy bridge was located between C9 and C10,
537 originally involved in a C=C bond. The production of epoxy fatty acids could be particularly
538 interesting for the involvement in a wide network of signaling lipids that generally regulate
539 inflammatory disease in humans (Kodani & Morisseau, 2019).

540 A single derivative of oleic acid was associated to the ion with m/z 297.2435, identified as 14-
541 hydroxy oleic acid. Interestingly, the latter is considered a potential antitumor agent, currently
542 undergoing clinical trials. Indeed, it induces cell cycle arrest, cellular differentiation, apoptosis,
543 and autophagy in a wide range of human cancer cells (Jang et al., 2017).

544 Ions compatible with the exact m/z values 311.2228 and 313.2384 were identified as
545 dihydroxylated derivatives of linoleic acid and oleic acid, respectively. In the former case one of
546 the hydroxylations always led to the displacement of one of the original C=C bonds of linoleic

acid, according to the process described before. As expected, this was not the case of oleic acid, whose dihydroxylated derivatives always retained the original C=C bond between C9 and C10. The production of dihydroxy fatty acids during lactic acid fermentation of walnuts appears useful and valuable since recently walnuts have attracted an increasing interest for multiple uses in industry (Kim & Oh, 2013), as well as in the pharmaceutical and medical fields (Kellerer et al., 2021). The production of hundreds of hydroxy fatty acids can be guaranteed by fatty acid hydroxylation enzymes, normally present in plants, animals and microorganisms. For instance, Nagatake & Kunisawa (2019) reconstructed the metabolic pathway for saturation of linoleic acid by *Lacti. plantarum*, leading to intermediates such as 10-hydroxy-cis-12-octadecenoic acid, 10-oxo-cis-12-octadecenoic acid, 10-oxo-trans-11-octadecenoic acid, 10-hydroxy-trans-11-octadecenoic acid, 10-oxo-octadecanoic acid 10-hydroxy-octadecanoic acid.

3.7. Trend of hydroxylated and epoxy fatty acids in walnut lipid extracts

Once five hydroxylated/epoxy fatty acids were identified by LC-MS/MS analysis, the second step of the investigation was focused on their different trend of production in walnuts fermented with LAB. As for MAG and DAG, the peak areas obtained from XIC chromatograms related to their ions were assumed to be proportional to the concentrations of the corresponding compounds and used as MS responses for a quantitative comparison, as summarized in Figure 6 and Figure S2. It is worth noting that peaks obtained from the extraction of ion currents for oxidized fatty acids were usually complex, due to the presence of isomeric compounds whose peaks could not be completely resolved by chromatography. Consequently, data reported in Figure 6 and Figure S2 represent the cumulative MS responses for isomeric compounds depicted in Figure 5.

569 Except for hydroxy-oleic fatty acid, the MS responses of oxidized species were significantly ($P <$
570 0.05) higher in Raw-Nuts compared to the controls Unstarted- and CA-Nuts. The gradual decrease
571 in the amount of these compounds during walnuts incubation at 30°C for 48 h can be traced back
572 to the temperature. In fact, many reactions underlying fat degradation (isomerization, oxidation,
573 *etc.*) might be favored by high temperatures (Brühl, et al., 2014). Regarding the fermented walnut,
574 it is worth noting that the MS responses of the detected dihydroxy fatty acids (dihydroxy oleic
575 [18:1] and dihydroxy linoleic [18:2] acid) were lower than those of the monohydroxy ones
576 (hydroxy oleic acid [18:1], hydroxy linoleic acid [18:2]/epoxy oleic acid [18:1], hydroxy linolenic
577 acid [18:3]). Assuming similar ionization yields for these compounds, these data suggest that lactic
578 fermentation of walnut promotes the synthesis and accumulation of the monohydroxylated forms
579 (Figure 6). Most of the fermented samples exhibited a decrease of the content of dihydroxy oleic
580 acid (18:1) and dihydroxy linoleic acid (18:2), with the only exception of *F. rossiae* 2MR6, which
581 caused a significant ($P < 0.05$) increase of the two compounds. To the best of our knowledge, no
582 previous studies explored lipid metabolism in *F. rossiae* under food-like conditions, although *F.*
583 *rossiae* was previously shown to accumulate timnodonic acid (corresponding to eicosapentaenoic
584 acid, EPA) during fermentation of *Portulaca oleracea* puree (Filannino et al., 2017). Walnuts
585 fermented with *Lev. brevis* DIV15 and *Lacti. plantarum* T1.3 showed the highest response for
586 hydroxy oleic acid (18:1), followed by walnuts fermented with *Lacti. pentosus* 03S8. To a lesser
587 extent, also *E. faecalis* KAFEPL63 and AVEL13, *Lc. lactis* UNIBZ23, *St. thermophilus* UNIBZ31
588 and UNIBZ81, *L. curvatus* PE5, *Leuc. mesenteroides* S3d1, *Lacti. plantarum* CB5 and AVEF17
589 led to an increase ($P < 0.05$) in hydroxy oleic acid (18:1) compared to Unstarted-, CA-, and Raw-
590 Nuts. Hydroxy linoleic acid 18:2/epoxy 18:1 and hydroxy linolenic acid (18:3) had an increasing
591 trend in most of fermented samples, with *W. cibaria* PEP23F as the main ($P < 0.05$) producer,

followed by *E. faecalis* KAFEPL63, *P. parvulus* S5w1, *Leuc. mesenteroides* S3d1, *L. paracasei* UNIBZ27 and AFII5, and *Lacti. plantarum* CB5 and AVEF17. Considering the MS responses reported in Figure 6, it is not surprising that high values were found for oxidized derivatives of the 18:2 (linoleic) acid, being this the most abundant fatty acid detected in raw walnuts (see Figure 2). On the other hand, it is interesting to note that, although the 18:3 (linolenic) acid was less abundant than the 18:2 one in raw walnuts, the MS response of its hydroxylated derivatives was often the highest one among oxidized fatty acids after fermentation with LAB. This finding might be related to the presence of a more unsaturated side chain in α -linolenic acid.

PCA based on the area of hydroxy/epoxy fatty acid derivatives in walnut samples (Figure S2) clearly shows, once again, that fatty acids metabolism is strongly LAB strain-dependent. Although the ability to produce hydroxylated derivatives from fatty acids was previously described for lactobacilli, *Staphylococcus* spp., *Enterococcus* spp., and *Pediococcus* spp., our study turns a spotlight on other neglected LAB, such as *W. cibaria* (Kim & Oh, 2013; Liang et al., 2020; Filannino et al., 2020; Rocchetti et al., 2021). A point that remains to be clarified in future investigations concerns the physiological significance of such enzymatic activities for LAB. Some authors suggested the hydroxylation of unsaturated fatty acids as a detoxification mechanism and survival strategy for bacteria living in fatty acid-rich environments, as in the case of phenolic compounds (Takeuchi et al., 2016). Other authors highlighted the antifungal potential of hydroxylated derivatives, which might translate into a competitive advantage for LAB (Ndagano et al., 2011).

4. Conclusions

Our pioneering study on the lactic fermentation of walnut sheds light on the lipid metabolism of a heterogeneous pool of lactic acid bacteria and opens up new perspectives on the processing of high-fat plant matrices, aimed at the development of novel, functional foods enriched in fatty acid derivatives with health-promoting effects. Certain strains of *W. cibaria*, *Leuc. Mesenteroides*, and *E. faecalis* emerged for the lipolytic activities and the ability to release hydroxy- and epoxy-fatty acids, in addition to the better-known lactobacilli. These metabolic traits were found to be strain-dependent, as different capabilities were observed in strains belonging to the same species. The differences did not appear to be related to various growth performances, but rather to the specific enzyme pattern exhibited by each strain during growth in walnut. Most of the oxidized derivatives identified during our study have not been previously reported in the literature. These findings make the selection of *ad-hoc* LAB cultures for tailored fermentations crucial.

Figure captions

Figure 1. (A) Microbiological and biochemical profile. Characterization of raw walnut (raw-nuts), walnut without microbial inoculum (unstarted-nuts), walnut without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and fermented-nuts with 31 strains of lactic acid bacteria (LAB), which were incubated for 48 h at 30°C. (B) Pseudo-heat map showing the microbiological (cell density of LAB, Log CFU g⁻¹), chemical (pH), and biochemical (concentration of carbohydrates and organic acids) characterization of raw-nuts, unstarted-nuts, CA-nuts and fermented-nuts. Rows are clustered using Euclidean distance and McQuitty linkage. The color scale shows the differences between the standardized data. Clusters (A-E) were recognized at the level of similarity marked by the orange vertical line.

Figure 2. Free fatty acids profile. Quantification of free fatty acids (mg g⁻¹ DM) through HPLC-HRMS in raw walnut (raw-nuts), walnut without microbial inoculum (unstarted-nuts), walnut without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and Fermented-Nuts with 31 strains of lactic acid bacteria, which were incubated for 48 h at 30°C. Data referred to bars labelled with different letters differ significantly (P < 0.05) (A).

Figure 3. Total Ion Current (TIC) chromatograms (retention time interval 0-65 min). TIC chromatograms were obtained by LC-HRMS analysis for the lipid extracts of raw- (panel A), unstarted- (panel B), CA- (panel C) and fermented walnut with *Weissella cibaria* PEP23F (panel D). Color code for detected lipid classes: yellow) hydroxylated/epoxidized fatty acids; green) main fatty acids and monoacylglycerols (MAG); blue) diacylglycerols (DAG). The sum compositions (total number of carbon atoms : total number of C=C bonds on the side chain/s) for some representative lipids are reported in bold.

Figure 4. Peak areas obtained from eXtracted Ion Current (XIC) chromatograms. XIC chromatograms for monoacylglycerols (MAG) and diacylglycerols (DAG) were obtained after HPLC-HRMS analysis of lipid extracts of raw walnut (raw-nuts), walnut without microbial inoculum (unstarted-nuts), walnut without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and fermented walnut with *Weissella cibaria* PEP23F (PEP23F), which were incubated for 48 h at 30°C. Bars with different superscript letters indicate peak areas differing significantly (P < 0.05).

Figure 5. Chemical structures hypothesized for precursor ions of hydroxylated/epoxidized fatty acids detected in lipid extracts of walnut samples and for diagnostic product ions detected in the respective MS/MS spectra. Exact *m/z* values are reported for all structures.

Figure 6. Values of peak areas obtained from eXtracted Ion Current (XIC) chromatograms referred to hydroxy/epoxy fatty acids. XIC chromatograms were obtained by HPLC-HRMS analysis of lipid extracts of raw walnut (Raw-Nuts), walnut without microbial inoculum (unstarted-nuts), walnut without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and fermented-nuts with 31 strains of lactic acid bacteria, which were incubated for 48 h at 30°C. Values associated to bars with different letters differ significantly ($P < 0.05$).

Supplementary material

Figure S1. Principal component analysis (PCA) biplot, based on free fatty acids concentration (mg g^{-1} DM) of raw-nuts, unstarted-nuts, CA-nuts and fermented-nuts (B).

Figure S2. Principal component analysis (PCA) biplot, based on hydroxy/epoxy fatty acids concentration (Unit) of raw-nuts, unstarted-nuts, CA-nuts and fermented-nuts (B).

Table S1. Cell density (Log CFU g^{-1}) of lactic acid bacteria, pH, and concentration (mg g^{-1} DM) of carbohydrates and organic acids of raw walnuts (raw-nuts), walnuts without microbial inoculum (unstarted-nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and fermented-nuts with 31 strains of lactic acid bacteria, which were incubated for 48 h at 30°C.

Table S2. Quantification of the main fatty acids (mg g^{-1} DM) in freeze-dried raw walnuts (raw-nuts), walnuts without microbial inoculum (unstarted-nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and fermented-nuts with 31 strains of lactic acid bacteria (LAB), which were incubated for 48 h at 30°C.

Table S3. Summary of MS-related data and possible sum compositions (total number of carbon atoms : total number of C=C bonds on the side chain/s) inferred for the putative monoacylglycerols (MAG) and diacylglycerols (DAG) detected in lipid extracts of raw walnuts (raw-nuts), walnuts without microbial inoculum (unstarted-nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-nuts) and fermented-nuts by LC-HRMS.

CRedit authorship contribution statement

Giuseppina Maria Fiorino: Investigation, Formal analysis, Writing - Original Draft. **Ali Zein Alabiden Tlais:** Investigation, Formal analysis, Writing - Original Draft. **Ilario Losito:** Methodology, Formal analysis, Writing - Review & Editing. **Pasquale Filannino:** Conceptualization, Methodology, Project administration, Writing - Review & Editing. **Marco Gobbetti:** Funding acquisition, Writing - Review & Editing. **Raffaella Di Cagno:** Conceptualization, Methodology, Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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885 **Table 1.** Lactic acid bacteria strains used in this study.

Species	Code	Source
<i>Lactiplantibacillus plantarum</i>	AVEF17	Avocado fruit
<i>Lactiplantibacillus plantarum</i>	T1.3	Date fruit
<i>Lactiplantibacillus plantarum</i>	CB5	Cheese
<i>Lactiplantibacillus plantarum</i>	DC400	Sourdough
<i>Lactiplantibacillus pentosus</i>	03S8	Olives
<i>Lactiplantibacillus pentosus</i>	0253	Olives
<i>Levilactobacillus brevis</i>	DIV15	<i>Drosophila melanogaster</i> digestive tract
<i>Levilactobacillus brevis</i>	MDI9	<i>D. melanogaster</i> digestive tract
<i>Furfurilactobacillus rossiae</i>	2MR6	Pineapple
<i>Furfurilactobacillus rossiae</i>	UNIBZ20	Sourdough
<i>Limosilactobacillus fermentum</i>	F1	French beans
<i>Limosilactobacillus fermentum</i>	UNIBZ15	Dairy product
<i>Latilactobacillus curvatus</i>	PE5	Peppers
<i>Lacticaseibacillus paracasei</i>	UNIBZ27	Cheese
<i>Lacticaseibacillus paracasei</i>	AFII5	Apple by-product
<i>Lacticaseibacillus paracasei/casei</i>	FM4	Apple
<i>Apilactobacillus kunkeei</i>	BV61	Honeybee (<i>Apis mellifera</i> L.) digestive tract
<i>Apilactobacillus kunkeei</i>	PL13	Bee-collected ivy pollen
<i>Leuconostoc mesenteroides</i>	S3d1	Sauerkraut
<i>Leuconostoc citreum</i>	S4d5	Sauerkraut
<i>Leuconostoc citreum</i>	S7d10	Sauerkraut
<i>Weissella cibaria</i>	PEP23F	Peppers
<i>Weissella cibaria</i>	P9	Papaya
<i>Streptococcus thermophilus</i>	UNIBZ31	Cheese
<i>Streptococcus thermophilus</i>	UNIBZ81	Cheese
<i>Lactococcus lactis</i>	AFII1	Apple by-product
<i>Lactococcus lactis</i>	UNIBZ23	Cheese
<i>Pediococcus parvulus</i>	S2w6	Sauerkraut
<i>Pediococcus parvulus</i>	S5w1	Sauerkraut
<i>Enterococcus faecalis</i>	AVEL13	Avocado fruit
<i>Enterococcus faecalis</i>	KAFEPL63	Prickly pear fruit

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Highlights

- Strain-specific bacterial lipolytic activities hydrolyzed triacylglycerols
- *W. cibaria* and *Leuc. mesenteroides* emerged for their lipolytic activities
- Twelve hydroxylated or epoxidized derivatives were identified, some unedited
- *W. cibaria* emerged for release of hydroxy 18:2/epoxy 18:1 and hydroxy 18:3

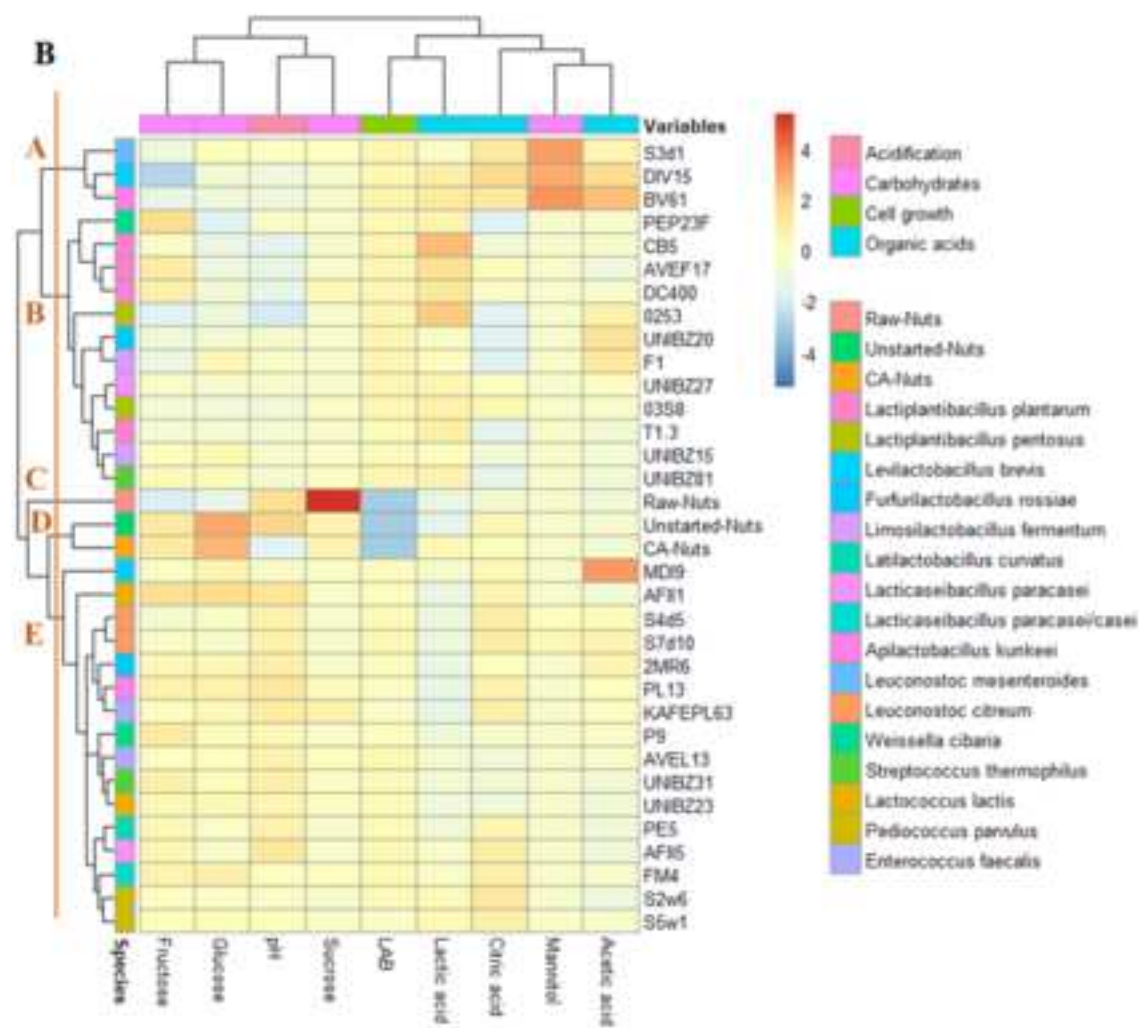
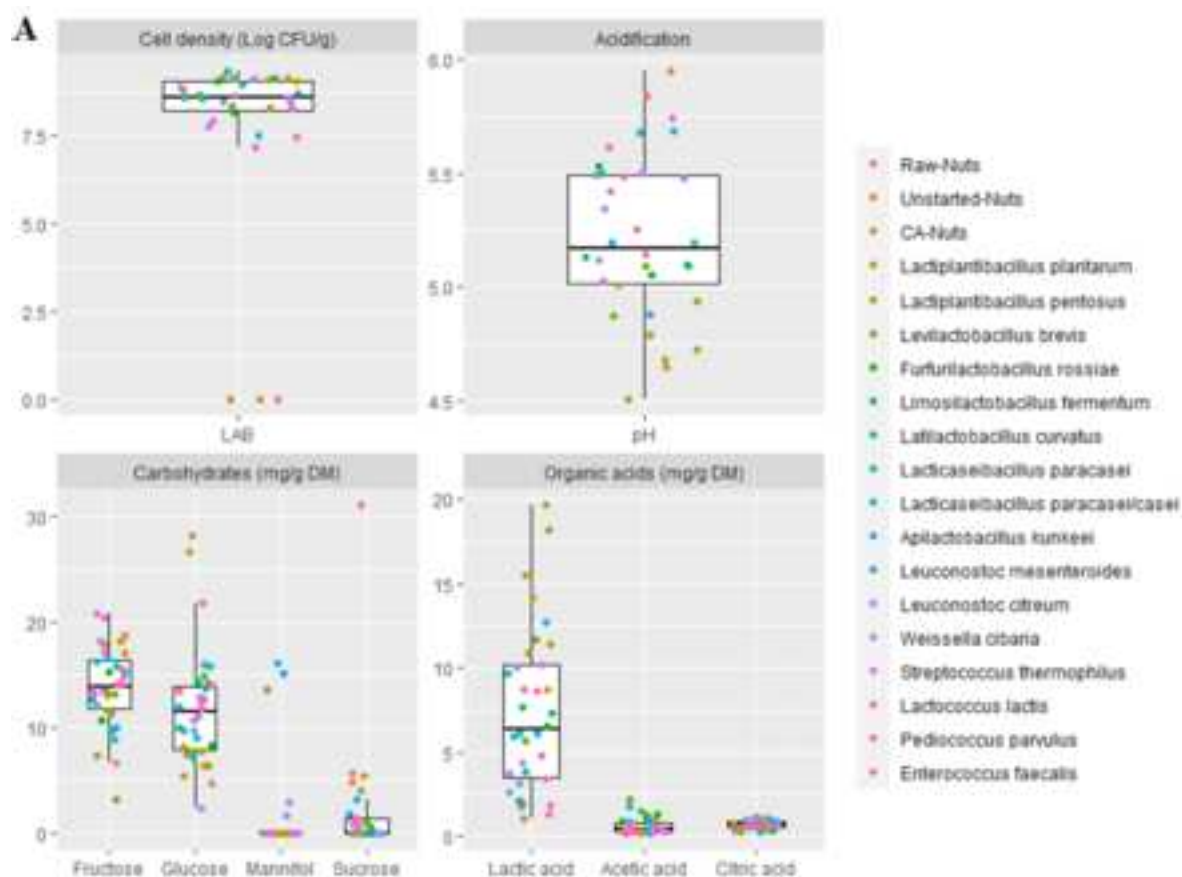


Figure 2

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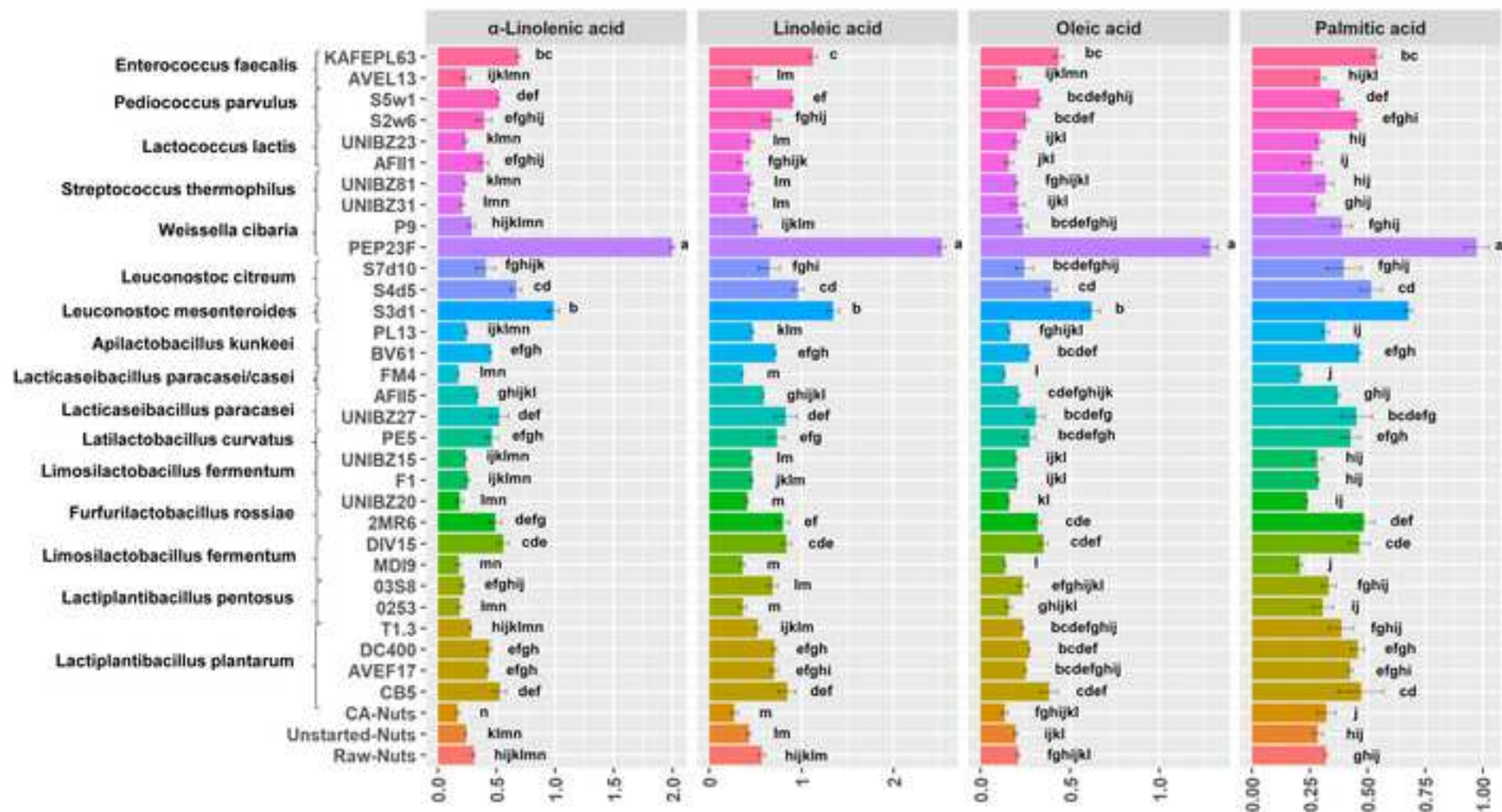


Figure 3

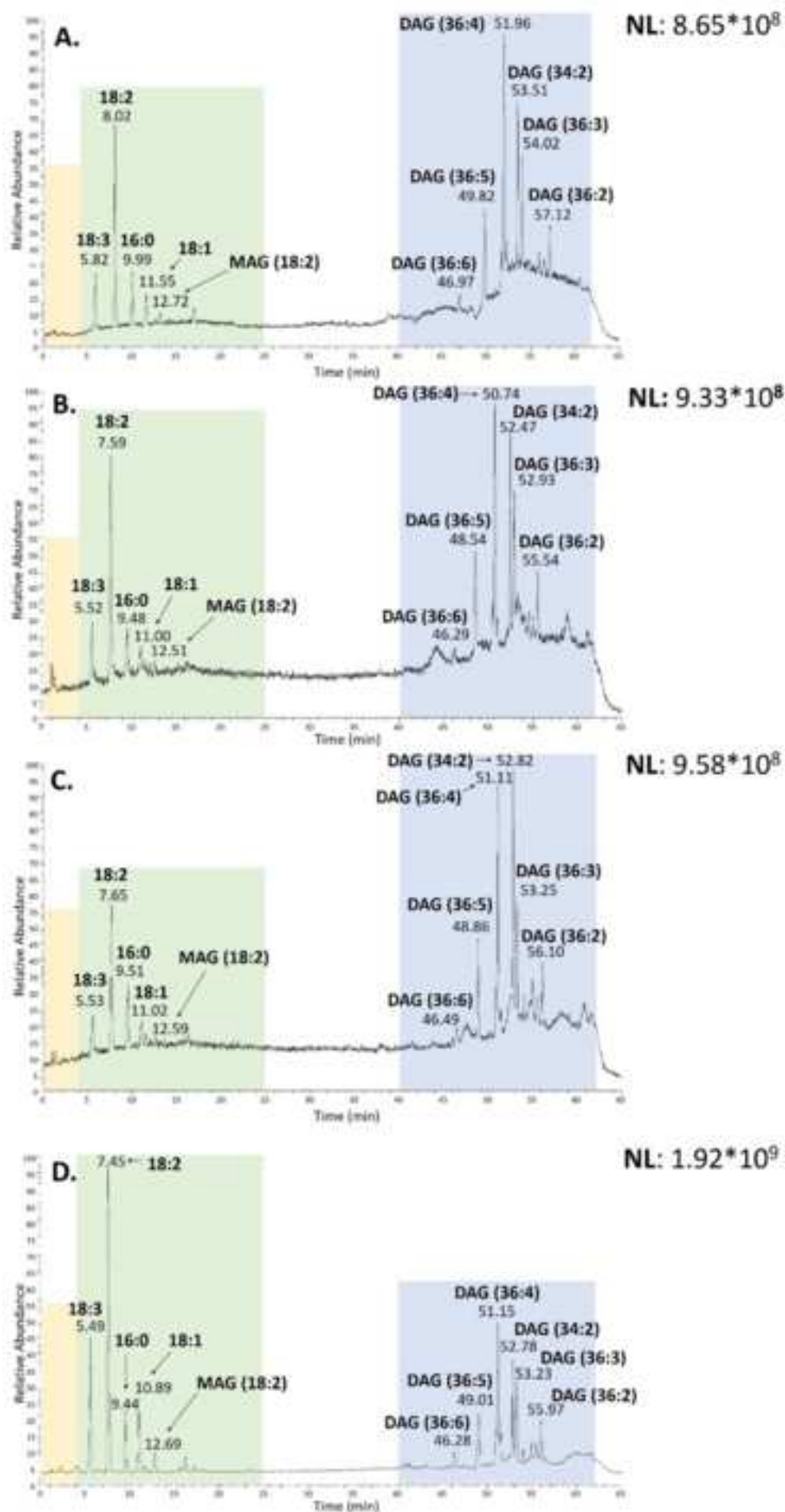
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Figure 4

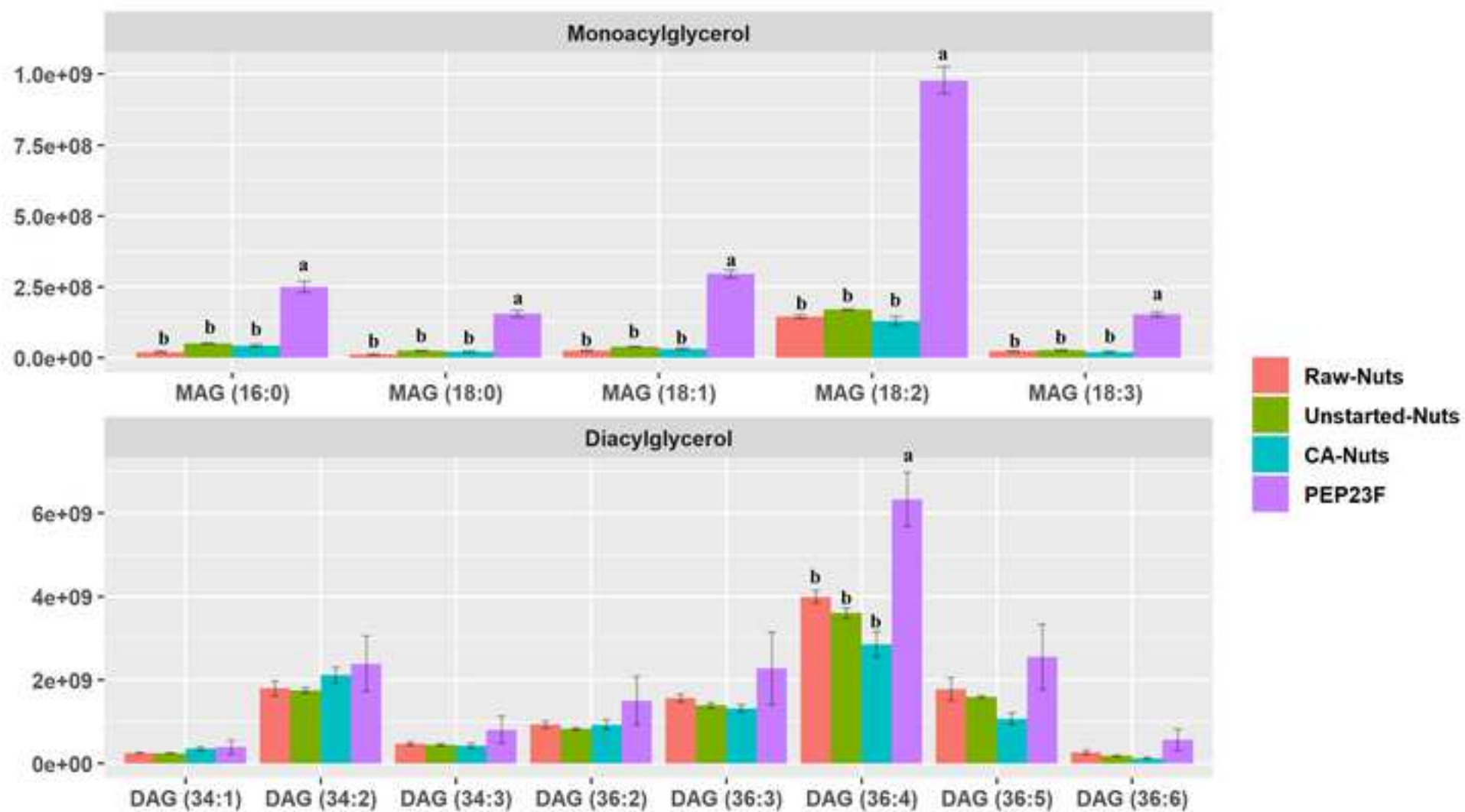


Figure 5

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Exact m/z	Identified compound	Diagnostic product ions detected in MS/MS spectra	Exact m/z	Identified compound	Diagnostic product ions detected in MS/MS spectra
293.2122	16-hydroxy- γ -linolenic acid (16-hydroxy-octadeca-6,9,12-trienoic acid)	 293.1704 121.1023 71.0602	297.2435	14-hydroxy-oleic acid (14-hydroxy-octadeca-9-enoic acid)	 183.1381 113.0872
	17-hydroxy- α -linolenic acid (17-hydroxy-octadeca-9,12,15-trienoic acid)	 323.1704 208.1547 127.0766 113.0806	311.2228	5,13-dihydroxy-octadeca-9,11-dienoic acid	 323.1704 87.0402
	9-hydroxy-octadeca-10,12,15-trienoic acid	 171.1027 87.0408 113.0872 82.0402		5,10-dihydroxy-octadeca-8,12-dienoic acid	 171.1027 87.0401 138.1128 87.0446
295.2279	13-hydroxy-octadeca-9,11-dienoic acid	 195.1381		10,16-hydroxy-octadeca-8,12-dienoic acid	 303.1809 235.1704
	15-hydroxy-linoleic acid (15-hydroxy-octadeca-9,12-dienoic acid)	 223.1704	313.2384	8,14-dihydroxy-oleic acid (8,14-dihydroxy-octadeca-9-enoic acid)	 225.1480 129.0821 86.0816
	9,10-epoxy-octadeca-12-enoic acid	 171.1027 127.1128 113.0872 87.0402		12,14-dihydroxy-oleic acid (12,14-dihydroxy-octadeca-9-enoic acid)	 183.1381 171.1381 98.0816

Figure 6

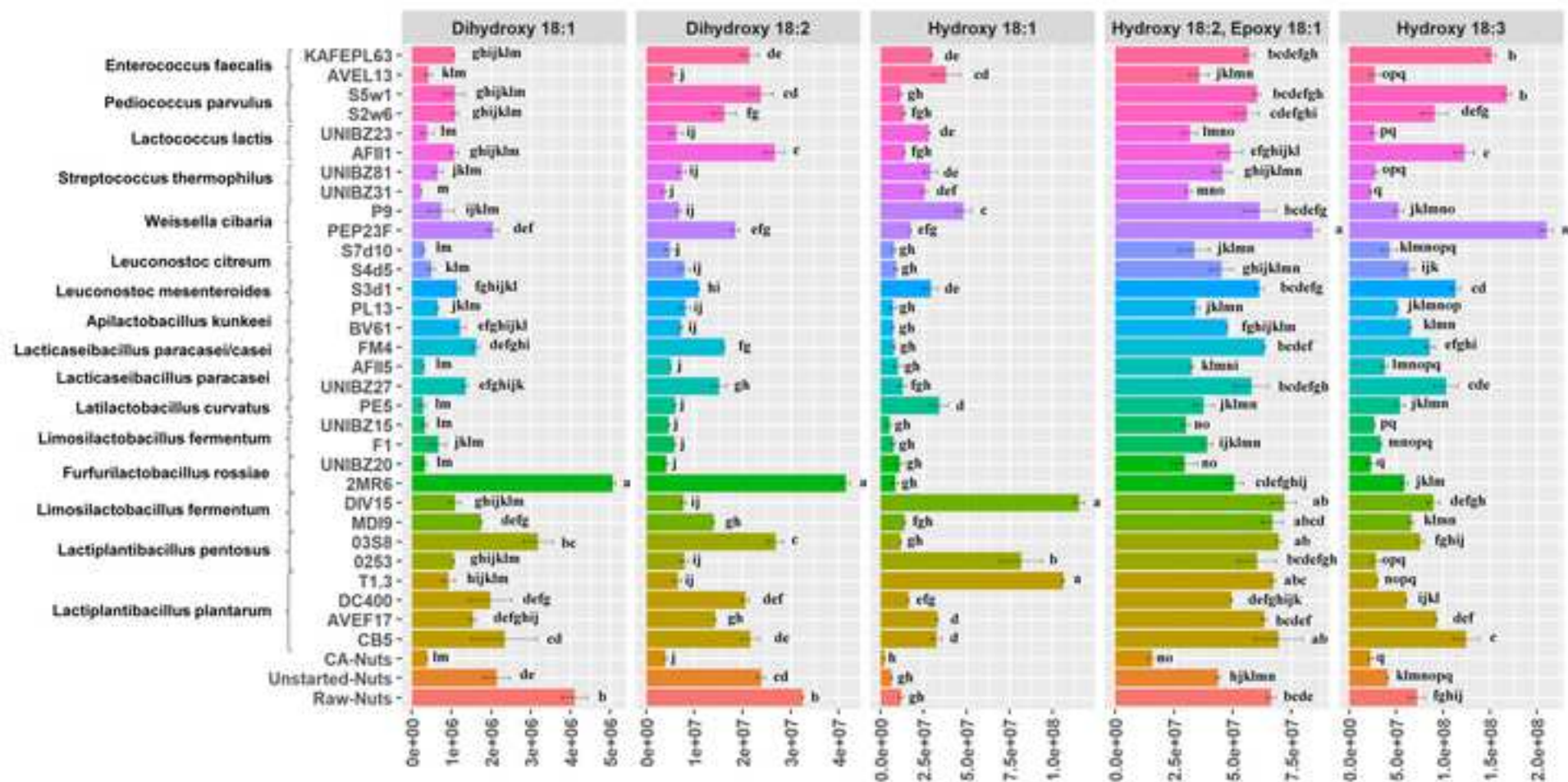
[Click here to access/download;Figure\(s\);Figure 6.tif](#)

Figure S1. Principal component analysis (PCA) biplot, based on free fatty acids concentration (mg g⁻¹ DM) of Raw-Nuts, Unstarted-Nuts, CA-Nuts and Fermented-Nuts (B).

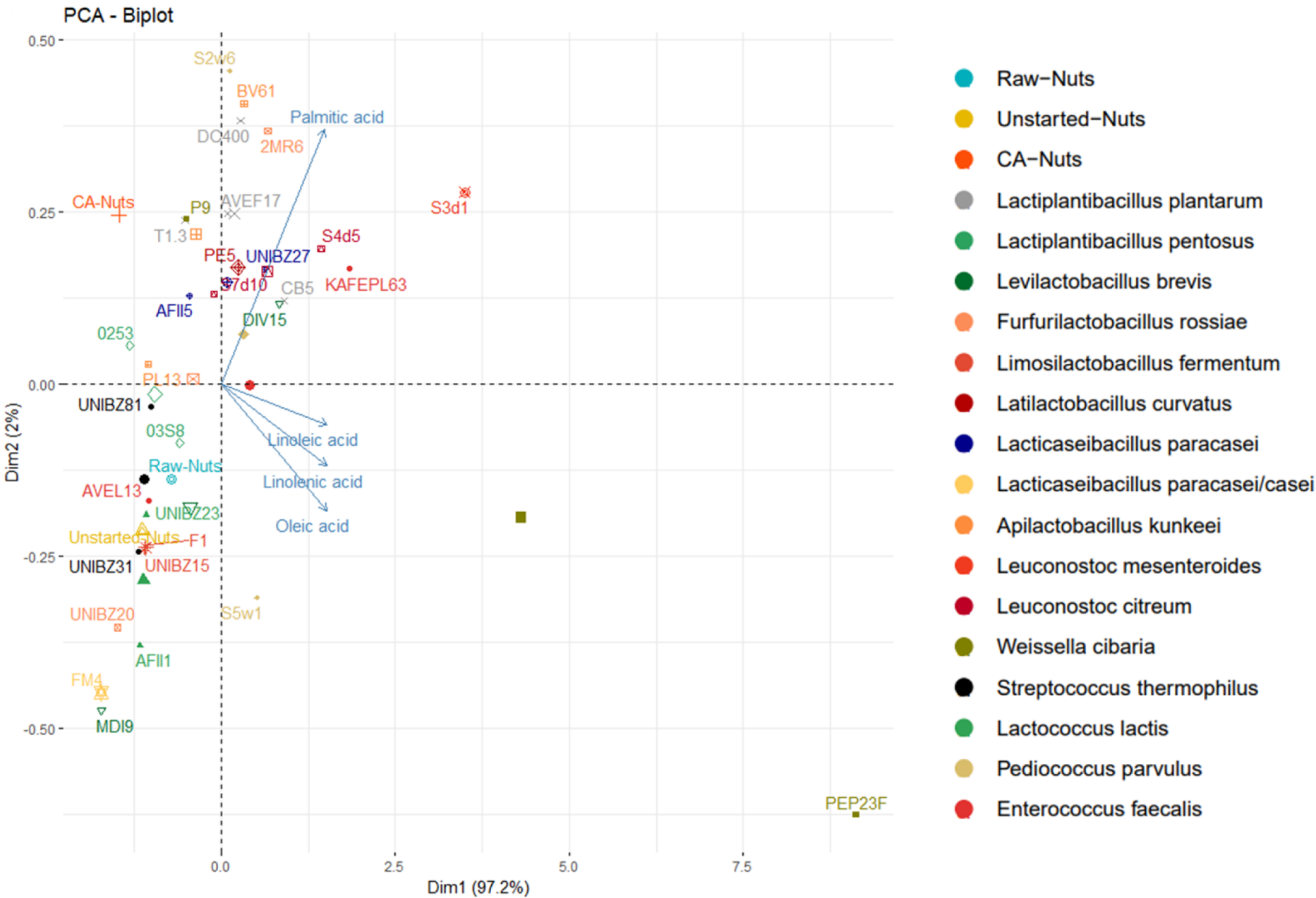


Figure S2. Principal component analysis (PCA) biplot, based on hydroxy/epoxy fatty acids concentration (Unit) of Raw-Nuts, Unstarted-Nuts, CA-Nuts and Fermented-Nuts (B).

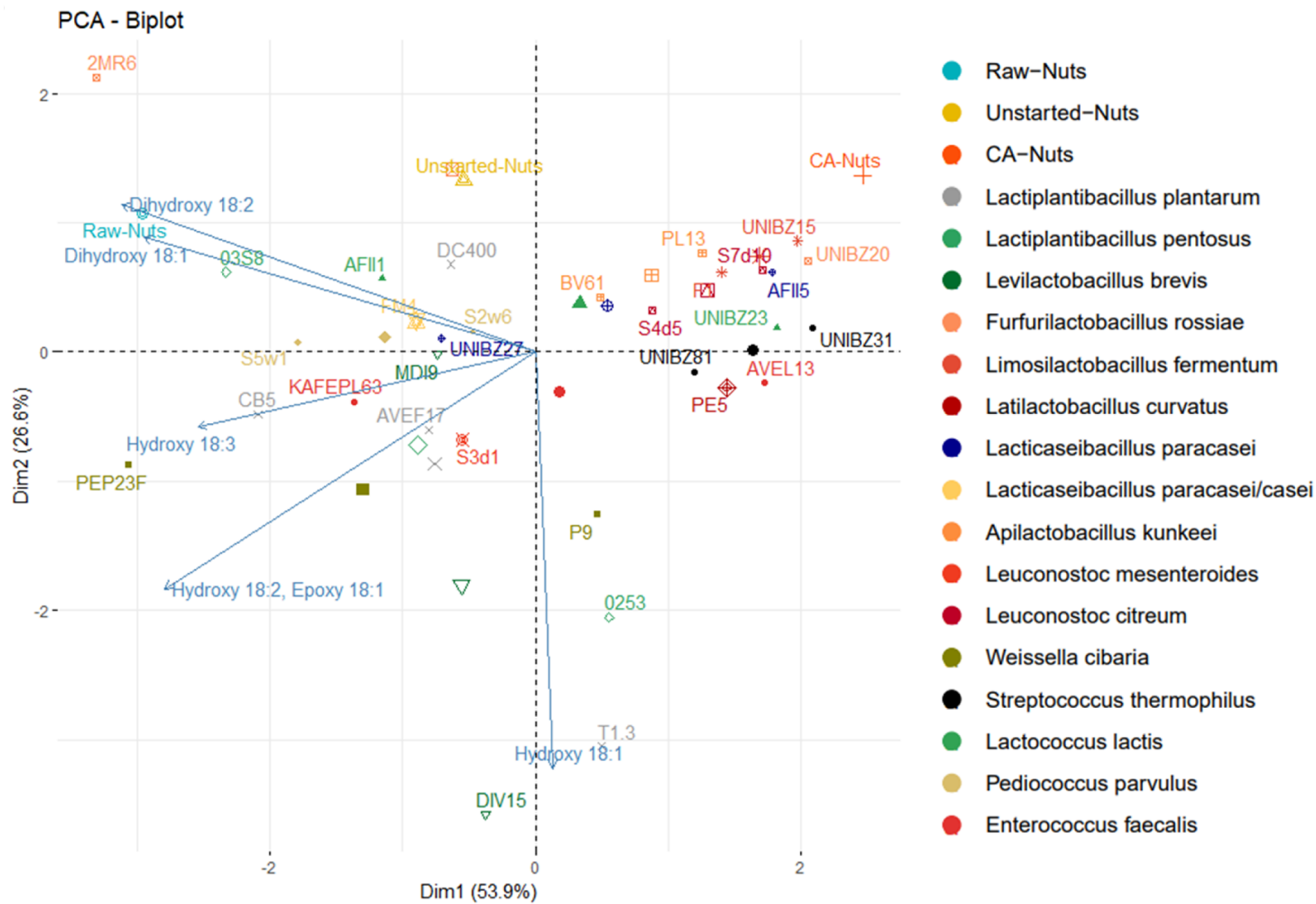


Table S1. Cell density (Log CFU g⁻¹) of lactic acid bacteria, pH, and concentration (mg g⁻¹ DM) of carbohydrates and organic acids of raw walnuts (Raw-Nuts), walnuts without microbial inoculum (Unstarted-Nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-Nuts) and Fermented-Nuts with 31 strains of lactic acid bacteria, which were incubated for 48 h at 30 °C.

Samples	Cell density (Log CFU g ⁻¹)	pH	Carbohydrates (mg g ⁻¹ DM)				Organic acids (mg g ⁻¹ DM)		
			Fructose	Glucose	Mannitol	Sucrose	Lactic acid	Citric acid	Acetic acid
Raw-Nuts	-	5.84 ± 0.01 ^b	6.67 ± 0.31 ⁿ	4.81 ± 0.22 ^{no}	-	31.13 ± 0.12 ^a	1.72 ± 0.02 ^{stu}	0.59 ± 0.01 ⁿ	0.22 ± 0.01 ^{fg}
Unstarted-Nuts (after incubation)	-	5.95 ± 0.03 ^a	18.84 ± 0.27 ^{abc}	28.19 ± 0.44 ^a	-	4.93 ± 0.33 ^{bc}	1.08 ± 0.02 ^u	0.82 ± 0.00 ^{hi}	0.36 ± 0.08 ^{fg}
CA-Nuts (after incubation)	-	4.65 ± 0.02 ^{cd}	18.25 ± 0.21 ^{abcd}	26.65 ± 0.77 ^{ab}	-	5.42 ± 0.45 ^{bc}	8.71 ± 0.29 ⁱ	0.32 ± 0.01 ^k	0.73 ± 0.03 ^{fg}
<i>Lactiplantibacillus plantarum</i> AVEF17	9.10 ± 0.09 ^{ab}	4.79 ± 0.10 ^s	17.92 ± 0.33 ^{abcd}	6.50 ± 0.19 ^{mno}	-	-	15.50 ± 0.01 ^c	0.74 ± 0.00 ^{kj}	0.15 ± 0.02 ^g
<i>Lacti. plantarum</i> T1.3	8.27 ± 0.06 ^{cdefg}	5.01 ± 0.04 ^{op}	11.37 ± 1.56 ^{hijkl}	8.27 ± 0.10 ^{klmno}	-	-	11.38 ± 0.05 ^{fg}	0.34 ± 0.00 ^{qp}	0.24 ± 0.00 ^{fg}
<i>Lacti. plantarum</i> CB5	9.02 ± 0.01 ^{abc}	4.68 ± 0.08 ^t	13.15 ± 0.27 ^{defghijk}	5.44 ± 0.3 ^{lmno}	-	-	19.66 ± 0.02 ^a	0.53 ± 0.00 ^m	0.41 ± 0.00 ^{fg}
<i>Lacti. plantarum</i> DC400	9.05 ± 0.03 ^{ab}	4.73 ± 0.01 ^s	17.08 ± 0.14 ^{abcde}	7.81 ± 0.75 ^{klmno}	-	4.07 ± 0.08 ^{cd}	14.18 ± 0.02 ^d	0.63 ± 0.00 ⁿ	0.39 ± 0.15 ^{fg}
<i>Lactiplantibacillus pentosus</i> 03S8	9.10 ± 0.15 ^{ab}	4.94 ± 0.04 ^{pq}	11.74 ± 0.29 ^{ghijkl}	7.58 ± 0.41 ^{lmno}	-	1.08 ± 0.36 ^{gfe}	10.90 ± 0.02 ^h	0.78 ± 0.02 ⁱ	0.39 ± 0.01 ^{fg}
<i>Lacti. pentosus</i> 0253	9.14 ± 0.11 ^{ab}	4.51 ± 0.01 ^u	7.47 ± 1.33 ^{klm}	6.49 ± 1.4 ^{lmno}	-	-	18.19 ± 0.05 ^b	0.28 ± 0.00 ^{sr}	0.97 ± 0.91 ^{cdef}
<i>Levilactobacillus brevis</i> MDI9	8.31 ± 0.21 ^{bcdef}	5.09 ± 0.01 ^{lmn}	13.21 ± 0.58 ^{defghijk}	14.87 ± 0.95 ^{nabcde}	-	0.80 ± 0.04 ^{gfe}	5.64 ± 0.02 ^l	0.79 ± 0.00 ⁱ	2.22 ± 0.03 ^a
<i>Lev. brevis</i> DIV15	8.65 ± 0.16 ^{abcde}	4.88 ± 0.08 ^r	3.30 ± 0.27 ^o	7.85 ± 0.12 ^{klmno}	13.65 ± 0.85 ^a	-	11.63 ± 0.01 ^f	1.15 ± 0.01 ^a	1.45 ± 0.42 ^{cd}
<i>Furfurilactobacillus rossiae</i> 2MR6	8.16 ± 0.09 ^{defgh}	5.53 ± 0.03 ^e	15.30 ± 0.32 ^{abcdefgh}	14.20 ± 0.00 ^{abcdef}	-	-	2.06 ± 0.00 ^{rs}	0.72 ± 0.00 ^{lk}	0.87 ± 0.03 ^{fg}
<i>F. rossiae</i> UNIBZ20	9.03 ± 0.18 ^{abc}	5.05 ± 0.03 ^{mno}	10.72 ± 0.39 ^{ijkl}	8.33 ± 0.57 ^{klmno}	-	-	6.57 ± 0.11 ^j	0.32 ± 0.00 ^{rq}	1.34 ± 0.06 ^{dc}
<i>Limosilactobacillus fermentum</i> F1	9.11 ± 0.10 ^{ab}	5.13 ± 0.04 ^{klm}	9.70 ± 0.27 ^{klm}	14.34 ± 0.20 ^{abcdef}	-	-	7.30 ± 0.12 ⁱ	0.28 ± 0.01 ^{sr}	1.19 ± 0.03 ^{edcb}
<i>Lim. fermentum</i> UNIBZ15	8.53 ± 0.14 ^{abcde}	5.09 ± 0.03 ^{lmn}	13.60 ± 0.16 ^{cdefghij}	13.75 ± 0.19 ^{abcdefg}	-	0.49 ± 0.08 ^{gfe}	7.67 ± 0.01 ⁱ	0.54 ± 0.00 ^m	0.29 ± 0.01 ^{fg}
<i>Latilactobacillus curvatus</i> PE5	8.56 ± 0.04 ^{abcde}	5.50 ± 0.01 ^{ef}	15.27 ± 0.23 ^{abcdefgh}	10.09 ± 0.72 ^{fghijklm}	-	-	3.86 ± 0.24 ^{no}	0.83 ± 0.00 ^{gi}	0.27 ± 0.01 ^{fg}
<i>Lacticaseibacillus paracasei</i> UNIBZ27	9.13 ± 0.02 ^{ab}	5.10 ± 0.03 ^{lmn}	12.74 ± 0.41 ^{efghijk}	9.08 ± 0.22 ^{hijklmn}	-	-	9.70 ± 0.03 ^h	0.68 ± 0.00 ^l	0.54 ± 0.25 ^{efg}
<i>L. paracasei</i> AFII5	8.71 ± 0.01 ^{abcd}	5.68 ± 0.00 ^{cd}	15.87 ± 0.25 ^{abcdefg}	9.80 ± 0.70 ^{hijklm}	-	-	5.90 ± 0.05 ^{kl}	0.95 ± 0.01 ^d	0.16 ± 0.03 ^g
<i>L. paracasei/casei</i> FM4	8.54 ± 0.14 ^{abcde}	5.20 ± 0.01 ^{ij}	16.30 ± 3.60 ^{abcdefg}	16.03 ± 0.86 ^{abcd}	-	1.46 ± 0.12 ^{fe}	6.12 ± 0.00 ^{kl}	0.91 ± 0.01 ^{ef}	0.41 ± 0.04 ^{cdefg}
<i>Apilactobacillus kunkeei</i> BV61	9.33 ± 0.17 ^a	4.88 ± 0.06 ^{qr}	8.89 ± 0.26 ^{lmn}	7.32 ± 0.15 ^{mno}	16.11 ± 0.27 ^a	0.00 ± 0.00 ^g	12.71 ± 0.03 ^e	1.02 ± 0.01 ^b	1.77 ± 0.09 ^{ba}
<i>A. kunkeei</i> PL13	7.40 ± 0.45 ^{ghi}	5.69 ± 0.05 ^{cd}	16.51 ± 2.98 ^{abcdef}	15.83 ± 3.56 ^{abcdef}	-	3.25 ± 0.86 ^d	1.88 ± 0.05 st	0.87 ± 0.01 ^{fg}	0.56 ± 0.03 ^{efg}
<i>Leuconostoc mesenteroides</i> S3d1	8.95 ± 0.02 ^{abc}	5.20 ± 0.04 ^{ijk}	10.07 ± 0.01 ^{jklm}	11.93 ± 1.18 ^{defghijk}	15.24 ± 3.32 ^a	1.82 ± 1.40 ^e	6.06 ± 0.07 ^{kl}	1.00 ± 0.01 ^{bc}	0.84 ± 0.01 ^{cdefg}
<i>Leuconostoc citreum</i> S4d5	8.45 ± 0.05 ^{abcde}	5.49 ± 0.08 ^{fg}	12.14 ± 0.62 ^{fghijkl}	11.14 ± 0.19 ^{efghijkl}	1.71 ± 0.12 ^{bc}	-	3.11 ± 0.01 ^{pq}	0.95 ± 0.00 ^{de}	0.65 ± 0.16 ^{defg}
<i>Leuc. citreum</i> S7d10	8.88 ± 0.02 ^{abcd}	5.48 ± 0.04 ^{efg}	13.26 ± 0.53 ^{defghijk}	9.83 ± 0.66 ^{ghijklm}	2.97 ± 0.70 ^b	-	2.65±0.00 ^{qr}	0.96 ± 0.02 ^{cd}	0.89 ± 0.01 ^{cdefg}
<i>Weissella cibaria</i> PEP23F	8.40 ± 0.04 ^{abcdef}	5.12 ± 0.03 ^{klm}	20.82 ± 0.26 ^a	2.37 ± 0.99 ^o	-	-	10.02 ± 0.57 ^h	0.25 ± 0.00 ^s	0.40 ± 0.00 ^{fg}
<i>W. cibaria</i> P9	7.74 ± 0.15 ^{fghi}	5.34 ± 0.01 ^h	18.27 ± 0.77 ^{abcd}	12.98 ± 3.11 ^{bcdefghij}	-	0.50 ± 0.32 ^{gfe}	3.75 ± 0.65 ^{nop}	0.61 ± 0.00 ⁿ	0.40 ± 0.01 ^{fg}
<i>Streptococcus thermophilus</i> UNIBZ31	8.57 ± 0.16 ^{abcde}	5.50 ± 0.04 ^{efg}	17.21 ± 0.72 ^{abcde}	12.88 ± 0.48 ^{bcdefghij}	-	0.91 ± 0.12 ^{gfe}	4.39 ± 0.05 ^{mn}	0.52 ± 0.00 ^{om}	0.27 ± 0.00 ^{fg}
<i>S. thermophilus</i> UNIBZ81	9.11 ± 0.17 ^{ab}	5.03 ± 0.03 ^{no}	14.78 ± 0.13 ^{bcdefghi}	12.55 ± 0.55 ^{cdefghij}	-	0.82 ± 0.24 ^{gfe}	10.19 ± 0.12 ^h	0.37 ± 0.01 ^p	0.23 ± 0.02 ^{fg}
<i>Lactococcus lactis</i> AFII1	7.89 ± 0.13 ^{efghi}	5.74 ± 0.07 ^c	20.40 ± 1.47 ^{ab}	21.82 ± 2.50 ^{abc}	-	0.05 ± 0.08 ^{gf}	1.86 ± 0.02 st	0.84 ± 0.01 ^{gi}	0.32 ± 0.02 ^{fg}
<i>Lc. lactis</i> UNIBZ23	8.27 ± 0.06 ^{cdefg}	5.42 ± 0.01 ^g	15.53 ± 0.39 ^{abcdefgh}	14.0 7± 0.32 ^{abcdef}	-	1.40 ± 0.04 ^{gfe}	3.43 ± 0.05 ^{op}	0.48 ± 0.01 ^o	0.18 ± 0.00 ^g
<i>Pediococcus parvulus</i> S2w6	8.59 ± 0.09 ^{abcde}	5.15 ± 0.01 ^{jkl}	13.49 ± 0.33 ^{defghijk}	10.78 ± 0.42 ^{fghijkl}	-	-	8.65 ± 0.01 ⁱ	1.01 ± 0.00 ^b	0.14 ± 0.01 ^g
<i>P. parvulus</i> S5w1	7.15 ± 0.2 ⁱ	5.25 ± 0.03 ⁱ	14.25 ± 1.88 ^{cdefghi}	12.50 ± 2.41 ^{cdefghij}	-	0.05 ± 0.07 ^{gf}	8.71 ± 0.02 ⁱ	0.90 ± 0.00 ^f	0.42 ± 0.01 ^{fg}
<i>Enterococcus faecalis</i> AVEL13	8.76 ± 0.06 ^{abcd}	5.49 ± 0.05 ^{efg}	13.66 ± 2.49 ^{cdefghij}	11.90 ± 1.18 ^{defghijk}	-	1.34 ± 0.80 ^{gfe}	4.77 ± 0.05 ^m	0.48 ± 0.01 ^o	0.41 ± 0.04 ^{fg}
<i>E. faecalis</i> KAFEPL63	7.45 ± 0.02 ^{hi}	5.62 ± 0.01 ^d	14.08 ± 0.50 ^{cdefghi}	13.50 ± 0.60 ^{abcdefgh}	-	5.80 ± 0.12 ^{bc}	1.34 ± 0.07 ^{tu}	0.89 ± 0.01 ^f	0.58 ± 0.00 ^{defg}

^{a–u} Means within the column with different letters are significantly different (P < 0.05).

(-): not detected.

Table S2. Quantification of the main fatty acids (mg g⁻¹ DM) in freeze-dried raw walnuts (Raw-Nuts), walnuts without microbial inoculum (Unstarted-Nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-Nuts) and Fermented-Nuts with 31 strains of lactic acid bacteria (LAB), which were incubated for 48 h at 30°C.

Samples	Linoleic acid (mg g ⁻¹ DM)	Linolenic acid (mg g ⁻¹ DM)	Oleic acid (mg g ⁻¹ DM)	Palmitic acid (mg g ⁻¹ DM)
Raw-Nuts	0.57 ± 0.03 ^{hijklm}	0.31 ± 0.01 ^{hijklmn}	0.21 ± 0.01 ^{fghijkl}	0.32 ± 0.01 ^{ghij}
Unstarted-Nuts (after incubation)	0.43 ± 0.02 ^{lm}	0.24 ± 0.00 ^{klmn}	0.20 ± 0.01 ^{ijkl}	0.29 ± 0.02 ^{hij}
CA-Nuts (after incubation)	0.27 ± 0.04 ^m	0.17 ± 0.02 ⁿ	0.13 ± 0.02 ^{fghijkl}	0.32 ± 0.04 ^j
<i>Enterococcus faecalis</i> AVEL13	0.47 ± 0.05 ^{lm}	0.24 ± 0.04 ^{ijklmn}	0.20 ± 0.02 ^{ijklmn}	0.30 ± 0.02 ^{hijkl}
<i>E. faecalis</i> KAFEPL63	1.13 ± 0.04 ^c	0.69 ± 0.02 ^{bc}	0.44 ± 0.03 ^{bc}	0.54 ± 0.02 ^{bc}
<i>Levilactobacillus brevis</i> DIV15	0.84 ± 0.05 ^{cde}	0.56 ± 0.05 ^{cde}	0.35 ± 0.03 ^{cdef}	0.47 ± 0.04 ^{cde}
<i>L. brevis</i> MDI9	0.36 ± 0.02 ^m	0.18 ± 0.02 ^{mn}	0.14 ± 0.00 ^l	0.21 ± 0.01 ^j
<i>Latilactobacillus curvatus</i> PE5	0.74 ± 0.09 ^{efg}	0.46 ± 0.06 ^{efgh}	0.27 ± 0.04 ^{bcdefgh}	0.43 ± 0.04 ^{efgh}
<i>Limosilactobacillus fermentum</i> UNIBZ15	0.46 ± 0.01 ^{lm}	0.24 ± 0.01 ^{ijklmn}	0.20 ± 0.01 ^{ijkl}	0.28 ± 0.03 ^{hij}
<i>L. fermentum</i> F1	0.47 ± 0.01 ^{mjklm}	0.25 ± 0.02 ^{ijklmn}	0.20 ± 0.01 ^{ijkl}	0.29 ± 0.00 ^{hij}
<i>Apilactobacillus kunkeei</i> BV61	0.72 ± 0.01 ^{efgh}	0.45 ± 0.00 ^{efgh}	0.27 ± 0.01 ^{bcdef}	0.46 ± 0.01 ^{efgh}
<i>Apilactobacillus kunkeei</i> PL13	0.47 ± 0.02 ^{klm}	0.25 ± 0.01 ^{ijklmn}	0.16 ± 0.00 ^{fghijkl}	0.32 ± 0.02 ^{ij}
<i>Lacticaseibacillus paracasei</i> UNIBZ27	0.83 ± 0.12 ^{def}	0.52 ± 0.08 ^{def}	0.31 ± 0.05 ^{bcdefg}	0.45 ± 0.07 ^{bcdefg}
<i>L. paracasei</i> AFH5	0.59 ± 0.00 ^{ghijkl}	0.34 ± 0.00 ^{ghijkl}	0.21 ± 0.01 ^{cdefghijk}	0.37 ± 0.01 ^{ghij}
<i>L. paracasei/casei</i> FM4	0.37 ± 0.00 ^m	0.18 ± 0.00 ^{lmn}	0.13 ± 0.00 ^l	0.21 ± 0.01 ^j
<i>Lactiplantibacillus pentosus</i> 0253	0.37 ± 0.04 ^m	0.19 ± 0.02 ^{lmn}	0.16 ± 0.02 ^{ghijkl}	0.31 ± 0.05 ^{ij}
<i>L. pentosus</i> 03-S-8	0.69 ± 0.06 ^{lm}	0.22 ± 0.01 ^{efghij}	0.24 ± 0.03 ^{efghijkl}	0.33 ± 0.03 ^{fghij}
<i>Lactiplantibacillus plantarum</i> CB5	0.85 ± 0.10 ^{def}	0.53 ± 0.06 ^{def}	0.38 ± 0.05 ^{cdef}	0.47 ± 0.10 ^{cd}

<i>L. plantarum</i> AVEF17	0.70 ± 0.04 ^{efghi}	0.43 ± 0.01 ^{efgh}	0.25 ± 0.01 ^{bcdefghij}	0.43 ± 0.01 ^{efghi}
<i>L. plantarum</i> DC400	0.71 ± 0.03 ^{efgh}	0.44 ± 0.02 ^{efgh}	0.27 ± 0.00 ^{bcdef}	0.46 ± 0.03 ^{efgh}
<i>L. plantarum</i> T1.3	0.53 ± 0.03 ^{ijklm}	0.28 ± 0.01 ^{hijklmn}	0.24 ± 0.01 ^{bcdefghij}	0.39 ± 0.05 ^{fghij}
<i>Furfurilactobacillus rossiae</i> UNIBZ20	0.41 ± 0.02 ^m	0.19 ± 0.03 ^{lmn}	0.16 ± 0.00 ^{kl}	0.24 ± 0.00 ^{ij}
<i>F. rossiae</i> 2MR6	0.80 ± 0.07 ^{ef}	0.49 ± 0.05 ^{defg}	0.32 ± 0.02 ^{cde}	0.49 ± 0.05 ^{def}
<i>Lactococcus lactis</i> AFII1	0.37 ± 0.06 ^{fghijk}	0.39 ± 0.04 ^{efghij}	0.16 ± 0.02 ^{ijkl}	0.26 ± 0.04 ^{ij}
<i>L. lactis</i> UNIBZ23	0.45 ± 0.04 ^{lm}	0.24 ± 0.02 ^{klmn}	0.20 ± 0.02 ^{ijkl}	0.29 ± 0.02 ^{hij}
<i>Leuconostoc citreum</i> S4d5	0.96 ± 0.06 ^{cd}	0.67 ± 0.05 ^{cd}	0.40 ± 0.03 ^{cd}	0.52 ± 0.05 ^{cd}
<i>Leuc. citreum</i> S7d10	0.65 ± 0.12 ^{fghi}	0.41 ± 0.09 ^{fghijk}	0.25 ± 0.05 ^{bcdefghij}	0.40 ± 0.08 ^{fghij}
<i>Leuconostoc mesenteroides</i> S3d1	1.35 ± 0.07 ^b	0.99 ± 0.05 ^b	0.62 ± 0.05 ^b	0.68 ± 0.01 ^b
<i>Pediococcus parvulus</i> S2w6	0.68 ± 0.10 ^{fghij}	0.40 ± 0.07 ^{efghij}	0.26 ± 0.02 ^{bcdef}	0.46 ± 0.01 ^{efghi}
<i>P. parvulus</i> S5w1	0.90 ± 0.01 ^{ef}	0.52 ± 0.01 ^{def}	0.33 ± 0.01 ^{bcdefghij}	0.38 ± 0.01 ^{def}
<i>Streptococcus thermophilus</i> UNIBZ31	0.42 ± 0.06 ^{lm}	0.21 ± 0.02 ^{lmn}	0.21 ± 0.04 ^{ijkl}	0.28 ± 0.02 ^{ghij}
<i>S. thermophilus</i> UNIBZ81	0.45 ± 0.03 ^{lm}	0.23 ± 0.01 ^{klmn}	0.20 ± 0.01 ^{fghijkl}	0.32 ± 0.04 ^{hij}
<i>Weissella cibaria</i> P9	0.53 ± 0.04 ^{ijklm}	0.29 ± 0.03 ^{hijklmn}	0.24 ± 0.03 ^{bcdefghij}	0.39 ± 0.04 ^{fghij}
<i>W. cibaria</i> PEP23F	2.53 ± 0.05 ^a	2.00 ± 0.02 ^a	1.29 ± 0.04 ^a	0.97 ± 0.05 ^a

^{a-m} Means within the column with different letters are significantly different (P < 0.05).

Table S3. Summary of MS-related data and possible sum compositions (total number of carbon atoms : total number of C=C bonds on the side chain/s) inferred for the putative monoacylglycerols (MAG) and diacylglycerols (DAG) detected in lipid extracts of raw walnuts (Raw-Nuts), walnuts without microbial inoculum (Unstarted-Nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-Nuts) and Fermented-Nuts by LC-HRMS.

Accurate <i>m/z</i>	Exact <i>m/z</i>	Mass accuracy (ppm)	R.T.	Composition
411.2754	411.2752	0.49	9.33-9.80	MAG (18:3)
413.2911	413.2909	0.36	12.50-12.72	MAG (18:2)
389.2912	389.2909	0.64	15.20-15.90	MAG (16:0)
415.3068	415.3065	0.60	16.94-17.69	MAG (18:1)
417.3225	417.3222	0.60	23.07-23.99	MAG (18:0)
671.4899	671.4892	1.04	46.15-46.97	DAG (36:6)
673.5051	673.5049	0.30	48.54-49.82	DAG (36:5)
649.5054	649.5049	0.77	50.47-51.68	DAG (34:3)
675.5207	675.5205	0.30	50.74-51.96	DAG (36:4)
651.5209	651.5205	0.61	52.47-53.51	DAG (34:2)
677.5363	677.5362	0.15	53.93-54.02	DAG (36:3)
653.5367	653.5362	0.69	54.54-55.92	DAG (34:1)
679.5522	679.5518	0.52	55.54-57.12	DAG (36:2)

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