Food Chemistry

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

--Manuscript Draft--

Manuscript Number:	FOODCHEM-D-22-08919R1
Article Type:	Research Article (max 7,500 words)
Keywords:	Lipolysis; hydroxy fatty acids; epoxy-fatty acids; fermentation; lactic acid bacteria; walnuts
Corresponding Author:	PASQUALE FILANNINO, Ph.D. University of Bari Aldo Moro Bari, ITALY
First Author:	Giuseppina Maria Fiorino
Order of Authors:	Giuseppina Maria Fiorino
	Ali Zein Alabiden Tlais
	Ilario Losito
	PASQUALE FILANNINO, Ph.D.
	Marco Gobbetti
	Raffaella Di Cagno
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.
Suggested Reviewers:	Emanuele Zannini University College Cork e.zannini@ucc.ie Food chemistry expertise
	LUCA SETTANNI University of Palermo luca.settanni@unipa.it Fermentation expertise
	Rudi Vogel Technical University of Munich rudi.vogel@wzw.tum.de Fermentation and Food chemistry expertise

Bari, 16th January 2023

Dear Editor,

I would like to thank you and the referee for giving us the opportunity to improve the manuscript. Please, note that all the recommendations, none excluded, have been considered in the revised version. An itemized list of the revisions according to the referee's recommendations has been provided.

Neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

Kind regards,

Pasquale Filannino

Dear Editor,

I would like to thank you and the referee for giving us the opportunity to improve the manuscript. Please, note that all the recommendations, none excluded, have been considered in the revised version. An itemized list of the revisions according to the referee's recommendations has been provided.

Kind regards,

Pasquale Filannino

Point by point response to reviewers:

Reviewer #2: The study is very interesting and worth investigating. However, the significance of the study must be highlighted. In addition, the relation of the used strains to in vitro or in vivo processes must be developed, e.g. the hypothetic processes by human gut microbiota. Please highlight health-promoting effects of fatty acids derivatives. **Ok**, the significance has been better emphasised (see P5 L103-105; P28 L614-615; P28 L622-623). In addition, the health-promoting effects of fatty acids derivatives, the role of the gut microbiome and probiotics has been highlighted (see P4 L72-85).

80 Please develop the abbreviation TAG and avoid abbreviation at the begining of sentences. **Ok**, it has been revised (see P5 L108).

Please avoid "lactobacilli"; it is suggested to be more precise and refer to *Lactobacillus* spp. Regarding this comment, I would like to underline that in 2020, the genus *Lactobacillus* has been reclassified into new 25 genera (Zheng et al., 2020). According to the new (and scientifically accepted) taxonomic classification, the current *Lactobacillus* genus includes only a small portion of the species initially included in the emended *Lactobacillus* genus and it would exclude many others (e.g. the crucial *Lactiplantibacillus plantarum*). Consequently, the community of microbiologists decided to use the term "lactobacilli" to "designate all organisms that were classified as *Lactobacillaceae* until 2020" (Zheng et al., 2020). Therefore, we prefer to keep the term 'lactobacilli', as the genus *Lactobacillus* does not include any of the species used in this study (according to the current and globally accepted taxonomic classification) (Zheng et al., 2020) and at the same time we are aligned with the scientific community.

For reference:
Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M., Harris, H. M., Mattarelli, P., ... & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. International journal of systematic and evolutionary microbiology, 70(4), 2782-2858.

L. 98 it is "lipid species", it should be "lipids". **Ok**, it has been revised (see P6 L130).

A list of chemicals used must be provided in the Materials and methods. **Ok**, the list of chemicals has been provided in Materials and methods section (see P6 L137; P7 L138-144).

L. 138 Please check "Lactococcus". We checked and we did not find any inconsistencies throughout the text.

Please use Greek letters for "alpha". It should be written in italics. **Ok**, it has been revised throughout the manuscript.

L. 163 "Eicosapentaenoic acid (EPA), cis-163 4,7,10, 13,16,19-Docosahexaenoic acid (DHA)" please use lowercase " acid (EPA), cis-163 4,7,10, 13,16,19-docosahexaenoic acid (DHA)." **Ok**, it has been done (see P6 L143-144).

L. 311-312 Please use the full names of species/strains instead of "Lacti." In general, to make an order, it is suggested to provide the table with strains used as starters in the manuscript's main text. Then, it is justified to use abbreviations. **OK**, Table S1 (showing the list of strains) has been moved in the manuscript's main text (please, see the new Table 1). Furthermore, species names in the manuscript are given in full the first time they are mentioned, and then in abbreviated form (please consider that this way is commonly followed by the scientific community in the microbiological field).

L. 333 I guess it should be α-linoleic acid. **You are right**, it has been revised (see P17 L377).

L. 370 I guess that the abbreviation LAB can be used here instead of "lactic acid bacteria". **Ok**, it has been revised (see P19 L415).

L. 433-436, L. 478-482 How did the Authors make such a conclusion based on MS analysis? The conclusion was based on the comparison between experimental m/z values and those reported at lines 475-476, that are the exact (theoretical) m/z vales expected for ions related to hydroxylated or epoxidized fatty acids with an appropriate number of C=C bonds.
This is a typical procedure performed when compounds for which some structural characteristics are known and have to be identified using high resolution MS data. First, molecular formulas of candidate compounds are considered (in this case those of different hydroxylated/epoxidized forms of fatty acids occurring in walnuts), the resulting exact m/z values are calculated and then a comparison is made with experimental m/z values.
A consistency between calculated and experimental m/z values on the third, or even on the fourth, decimal place was found in this case, as expected for the accuracy available with the adopted mass spectrometer, thus the hypothesized oxidized fatty acids were considered as good candidates to be further investigated using MS/MS analyses.
A more detailed explanation of the procedure followed to identify the three isomeric oxidized fatty acids detected at m/z 293.2122, starting from MS/MS data, has been reported in the revised version of the manuscript (see P23 L516-524; P24 L525-527).

In the highlights, there is a point "Strain-specific bacterial lipases hydrolyzed triacylglycerols" but I noted that this plot was not developed. Could you provide more data on the lipases theirselves? We agree with your observation. In fact, it is more appropriate to use the term 'lipase activity' rather than 'lipase', as we have not purified and characterized 'lipases', but we have conclusively demonstrated the presence of lipase activity. Consequently, the manuscript has been revised in accordance.

In general, in pdf files the resolution of all files is very poor, e.g. Figure 2A I could have not read the names of strains. I would like to explain that Elsevier's submission system inserts low-resolution images into the PDF, but provides a link for each image (at the top right of the PDF manuscript page) to download high-resolution figures (we must emphasise that we have uploaded very high-resolution images). In the high-resolution images, the names are perfectly readable. Furthermore, as the Figures have been rearranged (in accordance with the other reviewers' requests), some panels have been moved to the supplementary part, so that the remaining Figures in the main text are larger.

Table S1. I recommend using the full names. Please move the table to the main text. **OK**, table S1 has been moved in the manuscript's main text (please, see the new Table 1). Furthermore, species names in the manuscript are given in full the first time they are mentioned, and then in abbreviated form (this way is commonly followed by the scientific community).

Reviewer #3: The manuscript, entitled "Triacylglycerols hydrolysis and hydroxy- and epoxy-fatty acids release during fermentation of walnuts as model system: inter- and intra-species capabilities of lactic acid bacteria" (ID: FOODCHEM-D-22-08919), by Fiorino and other co-authors studied the behavior of 31 lactic acid bacteria strains was investigated during fermentation of Persian walnut. In spite of the work mentioned above, however, in my opinion, the manuscript needs some revisions and has several ambiguities.

I have some questions to discuss with the authors:
Firstly, why did the authors choose walnuts as fermentation substrate? According to the author's description in the manuscript, I understand that walnuts are rich in lipids. Was this the only reason? Was the walnut used as a substrate just for theoretical research? Did it have potential applications in the food industry? **Ok**, these points have been clarified in the revised manuscript (see P6 L122; P6 L126-128). Walnuts have been selected as a growth model substrate because of their peculiar lipid profile, but emerging evidence will gain industrial and functional relevance and facilitate the ad-hoc selection of starter cultures for fermentation of both walnuts-based products and other lipid-rich plant matrices.

Secondly, as far as I know, there have been some lactic acid fermentation in walnut research (such as, doi: 10.1016/j.lwt.2022.114204, doi: 10.1016/j.lwt.2022.113254), and the reports of lactic acid bacteria on fatty acids is also numerous. Would you explicitly specify the novelty of your work? What progress against the most recent state-of-the-art similar studies was made? Please, consider that our study is not limited to the effects of fermentation on free fatty acid concentration but it focuses on the production of hydroxy- and epoxy- derivatives. Regarding this last aspect, **to date there are no studies in the literature concerning the accumulation of hydroxy- and epoxy- derivatives throughout the fermentation of walnuts**. However, the two studies you cited (Mao et al. 2022 and Liu et al. 2022) have so little in common with our study.
According to Mao et al. 2022 (10.1016/j.lwt.2022.114204), (i) **the lactic acid bacteria they screened were unable to utilise triglycerides, so they had to add exogenous lipases to allow fatty acid conversion**; (ii) **the lactic acid bacteria they screened were not able to modify fatty acids, so they pursued their study with *Bifidobacterium breve*, which phylogenetically does not belong to the lactic acid bacteria group**; and (iii) **they did not mention hydroxy- and epoxy- derivatives**. On the contrary, we only used lactic acid bacteria (and not microorganisms from other groups), we did not use exogenous enzymes and we demonstrated how lactic acid bacteria are able to directly utilise triglycerides and convert free fatty acids into hydroxy- and epoxy- derivatives (this last result for food matrices is very innovative).
Liu et al. 2022 (10.1016/j.lwt.2022.113254) **only determined the free fatty acids profile during fermentation without mentioning any derivatives of lipid metabolism** (such as hydroxy- and epoxy- derivatives derivatives).
Therefore, we can confidently state that our study is not a replication of previous studies, and we have further emphasised this novelty in the introduction section (see P4 L92; P5 L93-97; P6 L124-126). To the best of our knowledge, most of the oxidized derivatives identified in our study had not previously been reported in the literature. Anyway, references to papers concerning oxidized fatty acids similar to those detected during our investigation have been provided in the manuscript.

In addition, please explain the reasons you chose these lactic acid bacteria. Did they have some functional or other commonalities or differences? Were there clear groupings? **Ok**, this point has been clarified in the revised manuscript (see P7 L150-153). To fully exploit the metabolic potential of the assorted group of lactic acid bacteria, the strains were chosen to ensure 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.

Finally, there were a lot of abbreviations. please add a list of abbreviations. **Ok**, the list of abbreviations has been provided (see P2 L41-46; P3 L47-49).

Title: The title did't seem to fit in well with the main point of the manuscript. **OK**, we have better focused the title according to the referee's request (please, see the new title).

Abstract: Abstract requires a revision to improve the comprehension of the work. Some sentences need rephrasing for a better clarity. **Ok**, the abstract has been revised. Please, see the new abstract.

Introduction:
Firstly, in the PDF version, there was only one paragraph. Reading it as a whole made readers very tired, and it was difficult to catch the author's key points. Could you consider presenting it in sections? Introduction can be improved in order to show better aim. Please carefully consider and revise the logic of some parts. Further, the study aim and background are not well presented, repetitions occurring in the paper should be avoided. **Ok**, the Introduction has been carefully revised based on your comments and divided into subsections.

Materials and methods:
Part of the method description was not clear and did not provide necessary references, please add. **Ok**, we have checked the Materials and methods section to ensure that all details are described and the necessary bibliographical references are given. If necessary, we ask the referee to specify which points are still unclear.

Line 149, providing only RPM was not scientific. **Ok**, it was reported as number of times the gravitational force [× g] (P9 L193).

2.5 Why were these 9 lipids measured, especially those that have no significance in the matrix. **Ok**, an explanation for the choice of the 9 fatty acid standards, including some that were not expected to be present in walnut, has been provided in the revised version of the paper (see P9 L206; P10 L207-211). Most standards were typical fatty acids occurring in walnuts, 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-eicosapentaenoic acid (EPA) and cis-4,7,10, 13,16,19-docosahexaenoic acid (DHA) were included among standards as models of polyunsaturated fatty acids, that might eventually be found in the samples of interest as a result of fermentation processes.

v: v or v/v? Please unify. **Ok**, it has been harmonised. We apologise for the inconsistencies.

Is there a space between ℃ the and the number? There are several inconsistencies in the text. **Ok**, it has been revised throughout the manuscript. We apologise for the inconsistencies.

Results and discussion:
Very conventional and unattractive. Please add some references especially in the last three years. We would like to point out that the journal Food Chemistry imposes a maximum article length (and therefore concise writing) and a limited number of references, so we had to select the references that we considered essential for understanding the manuscript. Out of about 30 references cited in Findings and Discussion, twelve are after 2020, ten are after 2017 and only 11 are before 2017 but are useful for the narrative.
We have also lightened the few parts that are difficult to read.

Figures:
Very bad. The picture was so vague that I can't get useful information. Please correct all of them! This comment appears cryptic to us. We do not understand what the referee means by "vague". It seems absurd to us that 'all' figures are 'vague', since we have used classical graphical tools (box plots, histograms, chromatograms) and statistical tools (Principal component analysis) commonly used in scientific publications. We could produce a very long list of bibliographic references (ours or those of other authors) in which the same graphic tools have been used.
In our humble opinion, the figures are understandable and rich in information. However, we have tried rearranging the figures, in order to give more space to the simpler figures and moving the more complex ones to the supplementary part (please see the new arrangement).

If the referee is referring to the resolution, we would like to point out that Elsevier's submission system inserts low resolution images into the PDF, but provide a link for each image (at the top right of the PDF manuscript page) to download the figures at high resolution (we must point out that we uploaded images at a very high resolution) (please see the above comment regarding the figure resolution).

Italicized legend label or not. **Ok**, it has been harmonised.

Figure 5 looks very much like a table. I would like to underline that Figure 5 looks like a table but it is not a table. It does not contain numerical results, but it is a graphical representation showing the chemical structures hypothesized for precursor ions of hydroxylated/epoxidized fatty acids. We do not think that the lines separating the chemical structures represent a weakness; on the contrary, the lines facilitate understanding. We do not understand what is the critical issue, and since we consider Figure 5 to be very clear and rich in information, we would like to keep it. Furthermore, even if we wanted to forcibly transform it into a table, this would also lead to considerable formatting problems for the Journal in proof editing.

RECEIVING EDITOR'S COMMENTS:

Address the issues raised by the reviewers; in addition to answering reviewers' or the editor's questions, the manuscript should be revised.

Highlights should showcase your results without the need to read the manuscript first and must have context and interest for readers; they must focus on the outcomes of your work. **Ok**, the Highlights were revised according to your suggestions.

- Behavior of 31 bacterial strains was investigated during fermentation of walnuts- what you did not what you found; focus on your results. **Ok**, it has been revised.

- Strain-specific bacterial lipases hydrolyzed triacylglycerols – what about them? Were found? Used? Lacks context. We agree with your observation. In fact, it is more appropriate to use the term "lipolytic activity" rather than "lipase", as we have not purified and characterized 'lipases', but we have conclusively demonstrated the presence of lipolytic activity. Consequently, the manuscript has been revised in accordance.

- Twelve hydroxylated or epoxidized derivatives were identified – new? To the best of our knowledge, most of the oxidized derivatives identified in our study had not been reported previously in the literature. Anyway, references to papers concerning oxidized fatty acids similar to those detected during our investigation were provided in the manuscript.

- Prominent activities emerged for W. cibaria, E. faecalis, and Leuc. Mesenteroides – and what are these activities? Lacks context. **Ok**, it has been revised.

The abstract should introduce your hypothesis, which can be further elaborated in the introduction. You launch in with what you did, not really explaining why. **Ok**, it has been revised. Please, see the new abstract.

25 Behavior of 31 lactic acid bacteria strains was investigated during fermentation of Persian walnut, which was selected as a model growth substrate due to its lipids content. – why? What's your hypothesis? **Ok**, it has been revised. Please, see the new abstract.

26 Most of the fermented walnut samples exhibited an increase in free fatty acids (linoleic, linolenic, palmitic, 28 and oleic acids). – content or type or both? Be more specific. **Ok**, the sentence has been revised to clarify that the content of those four fatty acids increased in most of the fermented walnut samples.

28 The increase of diacylglycerols and, especially, monoacylglycerols concentrations in fermented walnuts confirmed that strain-specific bacterial lipases hydrolyzed triacylglycerols during walnuts fermentation. – did it? Or did it confirm there is an association rather than causal relationship? The use of three controls (unfermented, unstarted and chemically acidified samples incubated under the same conditions of started samples) allows us to conclude with a good degree of confidence that the increase of diacylglycerols and, especially, monoacylglycerols concentrations in fermented walnuts confirmed that strain-specific bacterial lipolytic activities hydrolysed triacylglycerols during walnuts fermentation. We have some experience in managing lactic fermentation processes and we are able to identify (based on the experimental data) the mechanisms that drive changes in the composition of fermented matrices.

30 Twelve hydroxylated or epoxidized derivatives arising from oleic, linoleic, and linolenic fatty acids, in five groups of isomeric compounds, were also identified. A prominent role for Weissella cibaria, Enterococcus faecalis, and Leuconostoc mesenteroides in lipolytic activities including release of hydroxy- and epoxy fatty acids emerged, in addition to the better-known roles of some Lactobacilli spp. **Ok**, it has been partially revised following your suggestion. As already replied to the first referee, it is not correct to use the term *Lactobacillus* spp., but it is recommended to use the term "lactobacilli". In fact, in 2020, the genus *Lactobacillus* has been reclassified into new 25 genera and the community of microbiologists decided to use the term "lactobacilli" to "designate all organisms that were classified as *Lactobacillaceae* until 2020" (Zheng et al., 2020).

For reference:
Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M., Harris, H. M., Mattarelli, P., ... & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. International journal of systematic and evolutionary microbiology, 70(4), 2782-2858.

34 Such metabolic behaviors resulted in strain-dependent < what? word missing > since different capacities were observed among strains belonging to the same species. – that doesn't make much sense linguistically or scientifically. Revise. Also you should finish your abstract by responding to your hypothesis. **Ok**, it has been revised. Please, see the new abstract.

106 Thirty-one strains of lactic acid bacteria from the Culture Collection of the Department of. **Ok**, it has been revised (see P7 L147-149).

134 Ten grams of raw-, unstarted-, CA- and fermented nuts in sterile 0.9%. **Ok**, it has been revised (see P8 L178).

149 h, kept at 4 °C overnight, and centrifuged for 10 min at 10,000 rpm. – include the radius or (preferably) use x g. **Ok**, it was reported as number of times the gravitational force [× g]

Ensure all sources of reagents and equipment are included and compliant with the Guide for Authors, i.e., company name, city and country. **Ok**, a list of chemicals has been provided in Materials and Methods section (see P6 L137; P7 L138-144). In addition, information on equipment was provided throughout the manuscript.

There are issues with the English (syntax, grammar, etc.) throughout the manuscript. These must be addressed with the help of a native speaker or English language editing service, such as that provided by Elsevier https://webshop.elsevier.com/language-editing-services/ **Ok**, the manuscript has been revised by a colleague whose native language is English.

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

Giuseppina Maria Fiorino[a†], Ali Zein Alabiden Tlais[a†], Ilario Losito[b], Pasquale Filannino[c]*, Marco Gobbetti[a], Raffaella Di Cagno[a]

[a]Faculty of Sciences and Technology, Libera Università di Bolzano, 39100 Bolzano, Italy

[b]Department of Chemistry and SMART Inter-department Research Center, University of Bari Aldo Moro, 70126 Bari, Italy

[c]Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70126 Bari, Italy

[†]These authors have contributed equally to this work and share first authorship.

*Corresponding author. E-mail: pasquale.filannino1@uniba.it

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, docosahexaenoic 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 synthetized 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 ($HClO_4$), acetonitrile (CH_3CN, LC-MS grade), 2-propanol ($(CH_3)_2CHOH$, LC-MS grade), chloroform ($CHCl_3$, HPLC grade), methanol (CH3OH, LC-MS grade), ammonium acetate ($CH_3CO_2NH_4$) 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

161 Fresh walnut (*Juglans regia* L.) were purchased from a local supermarket in Bolzano, Italy. They
162 were shelled by hand, chopped into small pieces and blended with a Classic Blender (MB 550,
163 Microtron, Italy). Resulting homogenate, supplemented with sterile distilled water 10% (v w^{-1}),
164 was singly fermented by pure culture of lactic acid bacteria strains. Cells were cultivated in MRS
165 or M17 broth until the late exponential growth phase (ca. 24 h), washed twice in 50 mM phosphate
166 buffer, pH 7.0, and re-suspended into walnut homogenate at the initial cell density of ca. Log 7
167 CFU g^{-1}. After the inoculum, the fermentation was carried out at 30°C up to 48 h, leading to
168 samples defined as Fermented-Nuts. The time of fermentation and size of inoculum were defined
169 based on preliminary trials where growth and acidification capability of some lactic acid bacteria
170 strains were investigated (data not shown). Walnut without microbial inoculum (Unstarted-Nuts)
171 or chemically acidified with lactic acid (final pH of ca. 4.65) (CA-Nuts) were incubated under the
172 same conditions, representing the two controls. Samples before fermentation/incubation (Raw-
173 Nuts) were also considered. Before being subjected to compositional analysis, Raw-, Unstarted-,
174 CA- and Fermented-Nuts were directly frozen, then freeze-dried using a pilot scale lyophilizer
175 (Epsilon 2-6D LSC plus freeze-drier, Martin Christ, Osterode am Harz, Germany), and grounded.
176
177 **2.4. Microbiological analysis**
178 Ten grams of raw-, unstarted-, CA- and fermented nuts in sterile 0.9% (w v^{-1}) sodium chloride
179 solution were homogenized with a Classic Blender (PBI International Milan, Italy) for 2 min at
180 room temperature. Most LAB were enumerated on MRS agar, containing 0.1% of cycloheximide
181 (Sigma Chemical Co., Milan, Italy), at 30°C for 48 - 72 h under anaerobic conditions.
182 *Streptococcus* and *Lactococcus* species were enumerated on M17 agar, containing 0.1% of

183 cycloheximide (Sigma Chemical Co., Milan, Italy), at 37°C for 48 - 72 h under anaerobic
184 conditions.
185
186 **2.5. Physical and biochemical analyses**
187 The pH was measured by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland). In order to
188 determine the consumption of carbohydrates and the synthesis of organic acids during walnut
189 fermentation, freeze-dried walnut powder (2 g) was extracted with 20 mL of water/perchloric acid
190 (95:5, v:v) (Tlais et al., 2022). Mixture was sonicated (amplitude 60) using a macro-probe
191 sonicator (Vibra-Cell sonicator; Sonic and Materials Inc., Danbury, CT) for 1 min (2 cycles, 30 s
192 / cycle, 5 min interval between cycles) in an ice-bath. The suspension was subjected to stirring
193 conditions at 25°C for 1 h, kept at 4°C overnight, and centrifuged for 10 min at 11,000×g. Water-
194 soluble extracts (WSE) were filtered and stored at −20°C until further use. Concentrations of
195 glucose, fructose and mannitol were determined through a HPLC system Ultimate 3000 (Thermo
196 Fisher, Germering, Germany) equipped with a Spherisorb column (Waters, Millford, USA) and a
197 Perkin Elmer 200a refractive index detector (Tlais et al., 2022). Lactic, acetic and citric acids were
198 determined by LC-UV analysis, based on an Ultimate 3000 chromatograph (Thermo Fisher)
199 equipped with an Aminex HPX-87H column (ion exclusion, Biorad, Hercules, CA, USA) and a
200 UV detector operating at 210 nm (Tlais et al., 2022). Organic acid and sugar standards were
201 purchased from Sigma-Aldrich (Milan, Italy).
202
203 **2.6. Preparation of lipid standards**
204 Nine analytical standards, purchased by Sigma Aldrich (Milan, Italy), were used to develop the
205 LC-HRMS method to be subsequently used for the detection and quantification of fatty acids
206 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 $CH_3CN/(CH_3)_2CHOH/H_2O$ (65:30:5 v:v:v) mixture, with the exception of stearic and palmitic acids, that were dissolved in a $CHCl_3$/ CH_3OH (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 CH_3OH (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-, unstarted-, 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 $CHCl_3$ (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 CH_3OH (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-Exactive ™ 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, $1*10^6$ 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 Xcalibur™ v. 3.1 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for the control of the Q- Exactive plus™ 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 $[M + CH_3COO]^-$ 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 ± 0.44 mg g^{-1} DM) and CA-Nuts (26.65 ± 0.77 mg
349 g^{-1} DM). Similarly, high levels of fructose were found in Unstarted-Nuts (28.19 ± 0.44 mg g^{-1} DM)
350 and CA-Nuts (26.6 ± 0.77 mg g^{-1} DM), and walnuts fermented with *W. cibaria* strains (20.82 ±
351 0.26 and 18.27 ± 0.77 mg g^{-1} DM), and *Lc. Lactic* AFII1 (20.40 ± 1.47 mg g^{-1} 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 ± 0.01 mg g^{-1}
358 DM, respectively). The highest level of lactic acid was found in walnut fermented with *Lacti.*
359 *plantarum* strains (11.38 ± 0.01 – 19.66 ± 0.02 mg g^{-1} DM) followed by *Lacti. pentosus* strains
360 (10.90 ± 0.02 – 18.19 ± 0.02 mg g^{-1} DM) and *A. kunkeei* BV61 (12.71 ± 0.03 mg g^{-1} DM). Values
361 of acetic and citric acids were in the range 0.14 ± 0.01 – 2.22 ± 0.03 mg g^{-1} DM and 0.28 ± 0.00 –
362 1.15 ± 0.01 mg g^{-1} 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 ± 0.03 mg g^{-1} DM), followed by α-linolenic (0.31 ± 0.01 mg g^{-1} DM) and palmitic acids (0.32 ± 0.01 mg g^{-1} DM) and, at lower concentration, oleic acid (0.21 ± 0.01 mg g^{-1} 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 *Weissell*a,
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

411 involving saturated fatty acids. *Lim. fermentum* UNIBZ15 and F1 also showed overlapping fatty
412 acid profiles.
413
414 **3.4. Identification of TAG hydrolysis products in walnut lipid extracts by LC-HRMS**
415 Aiming at a better understanding about the lipid metabolism by LAB during the fermentation,
416 HRMS spectra underlying further peaks detected in LC-HRMS Total Ion Current (TIC)
417 chromatograms of fermented walnut extracts were carefully evaluated. As reported in the
418 literature, fatty acids are only a part of the total fat content in walnuts and other lipid classes can
419 be found, such as TAG (Fregapane et al., 2019). To verify if fatty acids found in fermented walnut
420 samples were also released from TAG, lipolysis by-products of the latter, namely DAG and MAG,
421 were searched for. The LC-HRMS TIC chromatograms obtained for the apolar fraction of Raw-
422 (panel A), Unstarted- (panel B), CA- (panel C) nuts and walnut fermented with *W. cibaria* PEP23F
423 (panel D) are reported in Figure 3. Fermentation with *W. cibaria* PEP23F was selected as a
424 representative condition because of the high content of free fatty acids (Figure 2 and Table S2),
425 which suggests a boosted hydrolysis occurring on TAG. As emphasized in Figure 3, MAG and
426 DAG species were identified in the four samples, following the procedure described in Section
427 2.8. Accurate *m/z* values enabled the retrieval of the sum compositions of those compounds (*i.e.*,
428 the total numbers of carbon atoms and C=C bonds located on their chain/chains). As expected,
429 DAG were eluted later than MAG from the C18 chromatographic column, due to their higher
430 hydrophobicity. Furthermore, for the same number of carbon atoms on the side chains, the
431 retention time decreased as the number of C=C bonds increases, due to the increase in polarity.
432 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

479 introduction of a OH group in place of a H atom linked to a C atom) and the epoxidation (the
480 introduction of a O atom as a "bridge" between two carbon atoms previously involved in a C=C
481 bond) of a specific fatty acid lead to compounds with the same molecular mass. In order to
482 distinguish these compounds and, more generally, to retrieve structural information on oxidized
483 fatty acids, MS/MS acquisitions were performed on the five precursor ions and the accurate *m/z*
484 values found for their most diagnostic product ions, along with the hypothesized chemical
485 structures, have been reported in Figure 5. As apparent, two or three isomeric species were
486 hypothesized for four of the five *m/z* values related to oxidized fatty acids, with differences
487 consisting in the position of the OH group(s) and of C=C bonds, according to the case.
488 The procedure followed to find the most likely chemical structures for oxidized fatty acids was
489 quite complex. Indeed, starting from major unsaturated fatty acids detected in walnut extracts, *i.e.*,
490 oleic, linoleic and α-linolenic ones, all possible locations of OH or epoxy functionalities on their
491 acyl chains were considered. Afterwards, fragmentation pathways were hypothesized for each
492 proposed structure, considering, as a guide, those previously observed for
493 hydroxylated/epoxidated fatty acids under similar collisional energy conditions (Losito et al.,
494 2018). As inferred from Figure 5, typical fragmentations involved C-C bond breakages, leading to
495 ionic fragments bearing a new C=C bond or a simple saturated C atom at one end of their structure.
496 Notably, the most common sites of chain breakage were close to carbon atoms involved in
497 hydroxylation or epoxidation, in accordance with previous findings (Losito et al., 2018). This
498 feature was fundamental to hypothesise the location of the OH group(s) or of the epoxy bridge,
499 according to the case. As also evidenced in Figure 5, fragmentation pathways were further
500 complicated by the possibility of intra-molecular charge transfer in precursor ions. Indeed, a proton
501 was occasionally found to be detached from a OH group, or even from a carbon atom located

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

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

Turning to specific structures reported for precursor ions in Figure 5, three isomeric compounds were found to be related to ions having *m/z* 293.2122, a value consistent with the occurrence of three C=C bonds and one OH groups on a side chain including 18 carbon atoms. After considering the locations of the three C=C bonds for α- or γ-linolenic acids, different possible locations for the OH group introduced upon fermentation and the structures of fragment ions obtained from MS/MS analyses, it was found that two of the three isomeric compounds corresponded, respectively, to γ-linolenic acid hydroxylated on C16 and α-linolenic acid hydroxylated on C17. MS/MS data obtained for the third compound were consistent with the structure of 9-hydroxy-octadeca-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

547 acid, according to the process described before. As expected, this was not the case of oleic acid,
548 whose dihydroxylated derivatives always retained the original C=C bond between C9 and C10.
549 The production of dihydroxy fatty acids during lactic acid fermentation of walnuts appears useful
550 and valuable since recently walnuts have attracted an increasing interest for multiple uses in
551 industry (Kim & Oh, 2013), as well as in the pharmaceutical and medical fields (Kellerer et al.,
552 2021). The production of hundreds of hydroxy fatty acids can be guaranteed by fatty acid
553 hydroxylation enzymes, normally present in plants, animals and microorganisms. For instance,
554 Nagatake & Kunisawa (2019) reconstructed the metabolic pathway for saturation of linoleic acid
555 by *Lacti. plantarum*, leading to intermediates such as 10-hydroxy-cis-12-octadecenoic acid, 10-
556 oxo-cis-12-octadecenoic acid, 10-oxo-trans-11-octadecenoic acid, 10-hydroxy-trans-11-
557 octadecenoic acid, 10-oxo-octadecanoic acid 10-hydroxy-octadecanoic acid.
558
559 **3.7. Trend of hydroxylated and epoxy fatty acids in walnut lipid extracts**
560 Once five hydroxylated/epoxy fatty acids were identified by LC-MS/MS analysis, the second step
561 of the investigation was focused on their different trend of production in walnuts fermented with
562 LAB. As for MAG and DAG, the peak areas obtained from XIC chromatograms related to their
563 ions were assumed to be proportional to the concentrations of the corresponding compounds and
564 used as MS responses for a quantitative comparison, as summarized in Figure 6 and Figure S2. It
565 is worth noting that peaks obtained from the extraction of ion currents for oxidized fatty acids were
566 usually complex, due to the presence of isomeric compounds whose peaks could not be completely
567 resolved by chromatography. Consequently, data reported in Figure 6 and Figure S2 represent the
568 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,

592 followed by *E. faecalis* KAFEPL63. *P. parvulus* S5w1, *Leuc. mesenteroides* S3d1, *L. paracasei*
593 UNIBZ27 and AFII5, and *Lacti. plantarum* CB5 and AVEF17. Considering the MS responses
594 reported in Figure 6, it is not surprising that high values were found for oxidized derivatives of the
595 18:2 (linoleic) acid, being this the most abundant fatty acid detected in raw walnuts (see Figure 2).
596 On the other hand, it is interesting to note that, although the 18:3 (linolenic) acid was less abundant
597 than the 18:2 one in raw walnuts, the MS response of its hydroxylated derivatives was often the
598 highest one among oxidized fatty acids after fermentation with LAB. This finding might be related
599 to the presence of a more unsaturated side chain in α-linolenic acid.
600 PCA based on the area of hydroxy/epoxy fatty acid derivatives in walnut samples (Figure S2)
601 clearly shows, once again, that fatty acids metabolism is strongly LAB strain-dependent. Although
602 the ability to produce hydroxylated derivatives from fatty acids was previously described for
603 lactobacilli, *Staphylococcus* spp., *Enterococcus* spp., and *Pediococcus* spp., our study turns a
604 spotlight on other neglected LAB, such as *W. cibaria* (Kim & Oh, 2013; Liang et al., 2020;
605 Filannino et al., 2020; Rocchetti et al., 2021). A point that remains to be clarified in future
606 investigations concerns the physiological significance of such enzymatic activities for LAB. Some
607 authors suggested the hydroxylation of unsaturated fatty acids as a detoxification mechanism and
608 survival strategy for bacteria living in fatty acid-rich environments, as in the case of phenolic
609 compounds (Takeuchi et al., 2016). Oher authors highlighted the antifungal potential of
610 hydroxylated derivatives, which might translate into a competitive advantage for LAB (Ndagano
611 et al., 2011).
612
613 **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.

Acknowledgements

This work was supported by the international program "HDHL INTIMIC-Knowledge Platform on food, diet, intestinal microbiomics and human health [DG DISR prot. N. 26406- 13.09.2018]". Title of the project: "Composizione nutrizionale e funzionale degli alimenti ed ontologia".

References

Ares-Yebra, A., Garabal, J. I., Carballo, J., & Centeno, J. A. (2019). Formation of conjugated linoleic acid by a Lactobacillus plantarum strain isolated from an artisanal cheese: evaluation in miniature cheeses. *International Dairy Journal*, *90*, 98-103. https://doi.org/10.1016/j.idairyj.2018.11.007

Black, B. A., Zannini, E., Curtis, J. M., & Gänzle, M. G. (2013). Antifungal hydroxy fatty acids produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal activity in bread. *Applied and Environmental Microbiology*, *79*(6), 1866-1873. https://doi.org/10.1128/AEM.03784-12

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911-917. https://doi.org/10.1139/o59-099

Brühl, L. (2014). Fatty acid alterations in oils and fats during heating and frying. *European Journal of Lipid Science and Technology*, *116*(6), 707-715. https://doi.org/10.1002/ejlt.201300273

Collins, Y. F., McSweeney, P. L., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal*, *13*(11), 841-866. https://doi.org/10.1016/S0958-6946(03)00109-2

Di Cagno, R., Filannino, P., & Gobbetti, M. (2017). Lactic acid fermentation drives the optimal volatile flavor-aroma profile of pomegranate juice. *International Journal of Food Microbiology*, *248*, 56-62. https://doi.org/10.1016/j.ijfoodmicro.2017.02.014

Di Cagno, R., Filannino, P., Cantatore, V., & Gobbetti, M. (2019). Novel solid-state fermentation of bee-collected pollen emulating the natural fermentation process of bee bread. *Food Microbiology*, *82*, 218-230. https://doi.org/10.1016/j.fm.2019.02.007

Di Cagno, R., Filannino, P., Vincentini, O., Lanera, A., Cavoski, I., & Gobbetti, M. (2016). Exploitation of *Leuconostoc mesenteroides* strains to improve shelf life, rheological, sensory and functional features of prickly pear (*Opuntia ficus-indica* L.) fruit puree. *Food Microbiology*, *59*, 176-189. https://doi.org/10.1016/j.fm.2016.06.009

Di Cagno, R., Minervini, G., Rizzello, C. G., De Angelis, M., & Gobbetti, M. (2011). Effect of lactic acid fermentation on antioxidant, texture, color and sensory properties of red and green smoothies. *Food Microbiology*, *28*(5), 1062-1071. https://doi.org/10.1016/j.fm.2011.02.011

Filannino, P., Cavoski, I., Thlien, N., Vincentini, O., De Angelis, M., Silano, M., Gobbetti, M., & Di Cagno, R. (2016). Lactic acid fermentation of cactus cladodes (*Opuntia ficus-indica* L.) generates flavonoid derivatives with antioxidant and anti-inflammatory properties. *PLoS One*, 11(3), e0152575. https://doi.org/10.1371/journal.pone.0152575

Filannino, P., Di Cagno, R., & Gobbetti, M. (2018). Metabolic and functional paths of lactic acid bacteria in plant foods: get out of the labyrinth. *Current Opinion in Biotechnology*, 49, 64-72. https://doi.org/10.1016/j.copbio.2017.07.016

Filannino, P., Di Cagno, R., Trani, A., Cantatore, V., Gambacorta, G., & Gobbetti, M. (2017). Lactic acid fermentation enriches the profile of biogenic compounds and enhances the functional features of common purslane (*Portulaca oleracea* L.). *Journal of Functional Foods*, *39*, 175-185. https://doi.org/10.1016/j.jff.2017.10.022

Filannino, P., Tlais, A. Z., Morozova, K., Cavoski, I., Scampicchio, M., Gobbetti, M., & Di Cagno, R. (2020). Lactic acid fermentation enriches the profile of biogenic fatty acid derivatives of avocado fruit (*Persea americana* Mill.). *Food Chemistry*, *317*, 126384. https://doi.org/10.1016/j.foodchem.2020.126384

749 Fiorino, G. M., Losito, I., De Angelis, E., Arlorio, M., Logrieco, A. F., & Monaci, L. (2019).
750 Assessing fish authenticity by direct analysis in real time-high resolution mass
751 spectrometry and multivariate analysis: Discrimination between wild-type and farmed
752 salmon. *Food Research International*, *116*, 1258-1265.
753 https://doi.org/10.1016/j.foodres.2018.10.013
754 Fregapane, G., Ojeda-Amador, R. M., & Salvador, M. D. (2019). Virgin walnut (*Juglans regia* L.)
755 oil. In *Fruit oils: Chemistry and Functionality* (pp. 133-147). Springer, Cham.
756 Fuka, M. M., Tanuwidjaja, I., Maksimovic, A. Z., Zunabovic-Pichler, M., Kublik, S., Hulak, N.,
757 Domig, K. J., & Schloter, M. (2020). Bacterial diversity of naturally fermented game meat
758 sausages: Sources of new starter cultures. *Lwt*, *118*, 108782.
759 https://doi.org/10.1016/j.lwt.2019.108782
760 Gangopadhyay, N. (2021). Oils Extracted from Nuts and Grains. *Oil and Oilseed Processing:*
761 *Opportunities and Challenges*, 61-79. https://doi.org/10.1002/9781119575313.ch4
762 Guasch-Ferré, M., Li, J., Hu, F. B., Salas-Salvadó, J., & Tobias, D. K. (2018). Effects of walnut
763 consumption on blood lipids and other cardiovascular risk factors: an updated meta-
764 analysis and systematic review of controlled trials. *The American Journal of Clinical*
765 *Nutrition*, 108(1), 174-187. https://doi.org/10.1093/ajcn/nqy091
766 Hira, T., Ogasawara, S., Yahagi, A., Kamachi, M., Li, J., Nishimura, S., Sakaino, M., Yamashita,
767 T., Kishino, S., Ogawa, J., & Hara, H. (2018). Novel mechanism of fatty acid sensing in
768 enteroendocrine cells: specific structures in oxo-fatty acids produced by gut bacteria are
769 responsible for CCK Secretion in STC-1 cells via GPR40. *Molecular Nutrition & Food*
770 *Research*, 62(19), 1800146. https://doi.org/10.1002/mnfr.201800146

771 Hosomi, K., Kiyono, H., Kunisawa, J. (2020). Fatty acid metabolism in the host and commensal
772 bacteria for the control of intestinal immune responses and diseases. *Gut Microbes*, *11*(3),
773 276-284. https://doi.org/10.1080/19490976.2019.1612662
774 Jang, E. J., Choi, W. R., Kim, S. Y., Hong, S. S., Rhee, I., Lee, S. J., Choi, S. W., Choi, H. G., &
775 Lim, S. J. (2017). 2-Hydroxyoleic acid-inserted liposomes as a multifunctional carrier of
776 anticancer drugs. *Drug Delivery*, 24(1), 1587-1597.
777 https://doi.org/10.1080/10717544.2017.1388452
778 Kellerer, T., Kleigrewe, K., Brandl, B., Hofmann, T., Hauner, H., & Skurk, T. (2021). Fatty acid
779 esters of hydroxy fatty acids (FAHFAs) are associated with diet, BMI, and age. *Frontiers*
780 *in Nutrition*, *8*, 436. https://doi.org/10.3389/fnut.2021.691401
781 Kim, K. R., & Oh, D. K. (2013). Production of hydroxy fatty acids by microbial fatty acid-
782 hydroxylation enzymes. *Biotechnology Advances*, *31*(8), 1473-1485.
783 https://doi.org/10.1016/j.biotechadv.2013.07.004
784 Kodani, S. D., & Morisseau, C. (2019). Role of epoxy-fatty acids and epoxide hydrolases in the
785 pathology of neuro-inflammation. *Biochimie*, *159*, 59-65.
786 https://doi.org/10.1016/j.biochi.2019.01.020
787 Li, J., Huang, Q., Zheng, X., Ge, Z., Lin, K., Zhang, D., Chen, Y., Wang, B., & Shi, X. (2020).
788 Investigation of the lactic acid bacteria in kazak cheese and their contributions to cheese
789 fermentation. *Frontiers in Microbiology*, *11*, 228.
790 https://doi.org/10.3389/fmicb.2020.00228
791 Liang, N., Tang, K., Curtis, J. M., & Gänzle, M. G. (2020). Identification and quantitation of
792 hydroxy fatty acids in fermented sausage samples. *Journal of Agricultural and Food*
793 *Chemistry*, 68(32), 8648-8657. https://doi.org/10.1021/acs.jafc.0c02688

Liu, W., Pu, X., Sun, J., Shi, X., Cheng, W., & Wang, B. (2022). Effect of *Lactobacillus plantarum* on functional characteristics and flavor profile of fermented walnut milk. *LWT*, 160, 113254. https://doi.org/10.1016/j.lwt.2022.113254

Losito, I., Facchini, L., Valentini, A., Cataldi, T. R., & Palmisano, F. (2018). Fatty acidomics: Evaluation of the effects of thermal treatments on commercial mussels through an extended characterization of their free fatty acids by liquid chromatography–Fourier transform mass spectrometry. *Food Chemistry*, *255*, 309-322. https://doi.org/10.1016/j.foodchem.2018.02.073

Mao, B., Guo, W., Huang, Z., Tang, X., Zhang, Q., Yang, B., Zhao, J., Cui, S., Zhang, H. (2022). Production of conjugated fatty acids in probiotic-fermented walnut milk with the addition of lipase. *LWT*, 172, 114204. https://doi.org/10.1016/j.lwt.2022.114204

McSweeney, P. L., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait*, *80*(3), 293-324. https://doi.org/10.1051/lait:2000127

Nagatake, T., Kunisawa, J. (2019). Emerging roles of metabolites of ω3 and ω6 essential fatty acids in the control of intestinal inflammation. *International Immunology*, *31*(9), 569-577. https://doi.org/10.1093/intimm/dxy086

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., & Thonart, P. (2011). Antifungal activity of 2 lactic acid bacteria of the Weissella genus isolated from food. *Journal of Food Science*, *76*(6), M305-M311. https://doi.org/10.1111/j.1750-3841.2011.02257.x

Paluchova, V., Vik, A., Cajka, T., Brezinova, M., Brejchova, K., Bugajev, V., Draberova, L., Draber, P., Buresova, J., Kroupova, P., Bardova, K., Rossmeisl, M., Kopecky, J., Hansen, T. V., Kuda, O. (2020). Triacylglycerol-rich oils of marine origin are optimal nutrients for

induction of polyunsaturated docosahexaenoic acid ester of hydroxy linoleic acid (13-DHAHLA) with anti-inflammatory properties in mice. *Molecular Nutrition & Food Research*, *64*(11), 1901238. https://doi.org/10.1002/mnfr.201901238

Pardatscher, R., & Schweigkofler, W. (2009). Microbial biodiversity associated with walnut *Juglans regia* L. in south Tyrol (Italy). *Mitt Klosterneuburg*, *59*, 17-23.

Porter, N.A., Caldwell, S.E., & Mills, K.A. (1995). Mechanisms of Free Radical Oxidation of Unsaturated Lipids. *Lipids*, *30*(4), 277-290. https://doi.org/10.1007/BF02536034

Pujo, J., Petitfils, C., Le Faouder, P., Eeckhaut, V., Payros, G., Maurel, S., Perez-Berezo, T., Van Hul, M., Barreau, F., Blanpied, C., Chavanas, S., Van Immerseel, F., Bertrand-Michel, J., Oswald, E., Knauf, C., Dietrich, G., Cani, P. D., Cenac, N. (2021). Bacteria-derived long chain fatty acid exhibits anti-inflammatory properties in colitis. *Gut*, *70*(6), 1088-1097. http://dx.doi.org/10.1136/gutjnl-2020-321173

Qin, X., Li, P., Lu, S., Sun, Y., Meng, L., Hao, J., & Fan, S. (2020). Phosphoproteomic analysis of lettuce (*Lactuca sativa* L.) reveals starch and sucrose metabolism functions during bolting induced by high temperature. *Plos One*, *15*(12), e0244198. https://doi.org/10.1371/journal.pone.0244198

Rocchetti, G., Rebecchi, A., Dallolio, M., Braceschi, G., Domínguez, R., Dallolio, G., Trevisan, M., Lorenzo, J.M., Lucini, L. (2021). Changes in the chemical and sensory profile of ripened Italian salami following the addition of different microbial starters. *Meat Science*, 180, 108584. https://doi.org/10.1016/j.meatsci.2021.108584

Saini, R. K., & Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance—A review. *Life Sciences*, *203*, 255-267. https://doi.org/10.1016/j.lfs.2018.04.049

Schoeler, M., Caesar, R. (2019). Dietary lipids, gut microbiota and lipid metabolism. *Reviews in Endocrine and Metabolic Disorders*, *20*(4), 461-472. https://doi.org/10.1007/s11154-019-09512-0

Takeuchi, M., Kishino, S., Park, S. B., Hirata, A., Kitamura, N., Saika, A., & Ogawa, J. (2016). Efficient enzymatic production of hydroxy fatty acids by linoleic acid Δ9 hydratase from *Lactobacillus plantarum* AKU 1009a. *Journal of Applied Microbiology*, *120*(5), 1282-1288. https://doi.org/10.1111/jam.13088

Tlais, A. Z. A., Kanwal, S., Filannino, P., Albiac, M. A., Gobbetti, M., & Di Cagno, R. (2022). Effect of sequential or ternary starters-assisted fermentation on the phenolic and glucosinolate profiles of sauerkraut in comparison with spontaneous fermentation. *Food Research International*, *156*, 111116. DOI: 10.1016/j.foodres.2022.111116

Tlais, A. Z., Da Ros, A., Filannino, P., Vincentini, O., Gobbetti, M., & Di Cagno, R. (2021). Biotechnological re-cycling of apple by-products: A reservoir model to produce a dietary supplement fortified with biogenic phenolic compounds. *Food Chemistry*, *336*, 127616. https://doi.org/10.1016/j.foodchem.2020.127616

van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L., & van Geel-Schutten, I. G. (2006). Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, *70*(1), 157-176. https://doi.org/10.1128/MMBR.70.1.157-176.2006

Yang, B., Chen, H., Stanton, C., Ross, R. P., Zhang, H., Chen, Y. Q., & Chen, W. (2015). Review of the roles of conjugated linoleic acid in health and disease. *Journal of Functional Foods*, *15*, 314-325. https://doi.org/10.1016/j.jff.2015.03.050

Yang, X., Hu, W., Xiu, Z., Jiang, A., Yang, X., Ji, Y., Guan, Y., & Feng, K. (2020). Microbial dynamics and volatilome profiles during the fermentation of Chinese northeast sauerkraut by *Leuconostoc mesenteroides* ORC 2 and *Lactobacillus plantarum* HBUAS 51041 under different salt concentrations. *Food Research International*, *130*, 108926. https://doi.org/10.1016/j.foodres.2019.108926

Zhao, W., Liu, M., Qin, Y., Bing, H., Zhang, F., & Zhao, G. (2022). Characterization and functional of four mutants of hydroxy fatty acid dehydrogenase from *Lactobacillus plantarum* p-8. *FEMS Microbiology Letters*. https://doi.org/10.1093/femsle/fnac060

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

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

886

887

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

Giuseppina Maria Fiorino[a†], Ali Zein Alabiden Tlais[a†], Ilario Losito[b], Pasquale Filannino[c]*, Marco Gobbetti[a], Raffaella Di Cagno[a]

[a]Faculty of Sciences and Technology, Libera Università di Bolzano, 39100 Bolzano, Italy

[b]Department of Chemistry and SMART Inter-department Research Center, University of Bari Aldo Moro, 70126 Bari, Italy

[c]Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70126 Bari, Italy

[†]These authors have contributed equally to this work and share first authorship.

*Corresponding author. E-mail: pasquale.filannino1@uniba.it

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, docosahexaenoic 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 synthetized 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 ($HClO_4$), acetonitrile (CH_3CN, LC-MS grade), 2-propanol ($(CH_3)_2CHOH$, LC-MS grade), chloroform ($CHCl_3$, HPLC grade), methanol (CH3OH, LC-MS grade), ammonium acetate ($CH_3CO_2NH_4$) 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 $CH_3CN/(CH_3)_2CHOH/H_2O$ (65:30:5 v:v:v) mixture, with the exception of stearic and palmitic acids, that were dissolved in a $CHCl_3$/ CH_3OH (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 CH_3OH (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-, unstarted-, 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 $CHCl_3$ (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 CH_3OH (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-Exactive ™ 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, $1*10^6$ 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 Xcalibur™ v. 3.1 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for the control of the Q- Exactive plus™ 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 $[M + CH_3COO]^-$ 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 ± 0.44 mg g^{-1} DM) and CA-Nuts (26.65 ± 0.77 mg
349 g^{-1} DM). Similarly, high levels of fructose were found in Unstarted-Nuts (28.19 ± 0.44 mg g^{-1} DM)
350 and CA-Nuts (26.6 ± 0.77 mg g^{-1} DM), and walnuts fermented with *W. cibaria* strains (20.82 ±
351 0.26 and 18.27 ± 0.77 mg g^{-1} DM), and *Lc. Lactic* AFII1 (20.40 ± 1.47 mg g^{-1} 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 ± 0.01 mg g^{-1}
358 DM, respectively). The highest level of lactic acid was found in walnut fermented with *Lacti.*
359 *plantarum* strains (11.38 ± 0.01 – 19.66 ± 0.02 mg g^{-1} DM) followed by *Lacti. pentosus* strains
360 (10.90 ± 0.02 – 18.19 ± 0.02 mg g^{-1} DM) and *A. kunkeei* BV61 (12.71 ± 0.03 mg g^{-1} DM). Values
361 of acetic and citric acids were in the range 0.14 ± 0.01 – 2.22 ± 0.03 mg g^{-1} DM and 0.28 ± 0.00 –
362 1.15 ± 0.01 mg g^{-1} 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 ± 0.03 mg g^{-1} DM), followed by α-linolenic (0.31 ± 0.01 mg g^{-1} DM) and palmitic acids (0.32 ± 0.01 mg g^{-1} DM) and, at lower concentration, oleic acid (0.21 ± 0.01 mg g^{-1} 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 *Weissell*a,
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

411 involving saturated fatty acids. *Lim. fermentum* UNIBZ15 and F1 also showed overlapping fatty
412 acid profiles.
413
414 **3.4. Identification of TAG hydrolysis products in walnut lipid extracts by LC-HRMS**
415 Aiming at a better understanding about the lipid metabolism by LAB during the fermentation,
416 HRMS spectra underlying further peaks detected in LC-HRMS Total Ion Current (TIC)
417 chromatograms of fermented walnut extracts were carefully evaluated. As reported in the
418 literature, fatty acids are only a part of the total fat content in walnuts and other lipid classes can
419 be found, such as TAG (Fregapane et al., 2019). To verify if fatty acids found in fermented walnut
420 samples were also released from TAG, lipolysis by-products of the latter, namely DAG and MAG,
421 were searched for. The LC-HRMS TIC chromatograms obtained for the apolar fraction of Raw-
422 (panel A), Unstarted- (panel B), CA- (panel C) nuts and walnut fermented with *W. cibaria* PEP23F
423 (panel D) are reported in Figure 3. Fermentation with *W. cibaria* PEP23F was selected as a
424 representative condition because of the high content of free fatty acids (Figure 2 and Table S2),
425 which suggests a boosted hydrolysis occurring on TAG. As emphasized in Figure 3, MAG and
426 DAG species were identified in the four samples, following the procedure described in Section
427 2.8. Accurate *m/z* values enabled the retrieval of the sum compositions of those compounds (*i.e.*,
428 the total numbers of carbon atoms and C=C bonds located on their chain/chains). As expected,
429 DAG were eluted later than MAG from the C18 chromatographic column, due to their higher
430 hydrophobicity. Furthermore, for the same number of carbon atoms on the side chains, the
431 retention time decreased as the number of C=C bonds increases, due to the increase in polarity.
432 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

479 introduction of a OH group in place of a H atom linked to a C atom) and the epoxidation (the
480 introduction of a O atom as a "bridge" between two carbon atoms previously involved in a C=C
481 bond) of a specific fatty acid lead to compounds with the same molecular mass. In order to
482 distinguish these compounds and, more generally, to retrieve structural information on oxidized
483 fatty acids, MS/MS acquisitions were performed on the five precursor ions and the accurate *m/z*
484 values found for their most diagnostic product ions, along with the hypothesized chemical
485 structures, have been reported in Figure 5. As apparent, two or three isomeric species were
486 hypothesized for four of the five *m/z* values related to oxidized fatty acids, with differences
487 consisting in the position of the OH group(s) and of C=C bonds, according to the case.
488 The procedure followed to find the most likely chemical structures for oxidized fatty acids was
489 quite complex. Indeed, starting from major unsaturated fatty acids detected in walnut extracts, *i.e.*,
490 oleic, linoleic and α-linolenic ones, all possible locations of OH or epoxy functionalities on their
491 acyl chains were considered. Afterwards, fragmentation pathways were hypothesized for each
492 proposed structure, considering, as a guide, those previously observed for
493 hydroxylated/epoxidated fatty acids under similar collisional energy conditions (Losito et al.,
494 2018). As inferred from Figure 5, typical fragmentations involved C-C bond breakages, leading to
495 ionic fragments bearing a new C=C bond or a simple saturated C atom at one end of their structure.
496 Notably, the most common sites of chain breakage were close to carbon atoms involved in
497 hydroxylation or epoxidation, in accordance with previous findings (Losito et al., 2018). This
498 feature was fundamental to hypothesise the location of the OH group(s) or of the epoxy bridge,
499 according to the case. As also evidenced in Figure 5, fragmentation pathways were further
500 complicated by the possibility of intra-molecular charge transfer in precursor ions. Indeed, a proton
501 was occasionally found to be detached from a OH group, or even from a carbon atom located

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

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

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

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

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

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

Ions compatible with the exact *m/z* values 311.2228 and 313.2384 were identified as dihydroxylated derivatives of linoleic acid and oleic acid, respectively. In the former case one of 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,

592 followed by *E. faecalis* KAFEPL63. *P. parvulus* S5w1, *Leuc. mesenteroides* S3d1, *L. paracasei*
593 UNIBZ27 and AFII5, and *Lacti. plantarum* CB5 and AVEF17. Considering the MS responses
594 reported in Figure 6, it is not surprising that high values were found for oxidized derivatives of the
595 18:2 (linoleic) acid, being this the most abundant fatty acid detected in raw walnuts (see Figure 2).
596 On the other hand, it is interesting to note that, although the 18:3 (linolenic) acid was less abundant
597 than the 18:2 one in raw walnuts, the MS response of its hydroxylated derivatives was often the
598 highest one among oxidized fatty acids after fermentation with LAB. This finding might be related
599 to the presence of a more unsaturated side chain in α-linolenic acid.
600 PCA based on the area of hydroxy/epoxy fatty acid derivatives in walnut samples (Figure S2)
601 clearly shows, once again, that fatty acids metabolism is strongly LAB strain-dependent. Although
602 the ability to produce hydroxylated derivatives from fatty acids was previously described for
603 lactobacilli, *Staphylococcus* spp., *Enterococcus* spp., and *Pediococcus* spp., our study turns a
604 spotlight on other neglected LAB, such as *W. cibaria* (Kim & Oh, 2013; Liang et al., 2020;
605 Filannino et al., 2020; Rocchetti et al., 2021). A point that remains to be clarified in future
606 investigations concerns the physiological significance of such enzymatic activities for LAB. Some
607 authors suggested the hydroxylation of unsaturated fatty acids as a detoxification mechanism and
608 survival strategy for bacteria living in fatty acid-rich environments, as in the case of phenolic
609 compounds (Takeuchi et al., 2016). Oher authors highlighted the antifungal potential of
610 hydroxylated derivatives, which might translate into a competitive advantage for LAB (Ndagano
611 et al., 2011).
612

613 **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.

Acknowledgements

This work was supported by the international program "HDHL INTIMIC-Knowledge Platform on food, diet, intestinal microbiomics and human health [DG DISR prot. N. 26406- 13.09.2018]". Title of the project: "Composizione nutrizionale e funzionale degli alimenti ed ontologia".

References

Ares-Yebra, A., Garabal, J. I., Carballo, J., & Centeno, J. A. (2019). Formation of conjugated linoleic acid by a Lactobacillus plantarum strain isolated from an artisanal cheese: evaluation in miniature cheeses. *International Dairy Journal*, *90*, 98-103. https://doi.org/10.1016/j.idairyj.2018.11.007

Black, B. A., Zannini, E., Curtis, J. M., & Gänzle, M. G. (2013). Antifungal hydroxy fatty acids produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal activity in bread. *Applied and Environmental Microbiology*, *79*(6), 1866-1873. https://doi.org/10.1128/AEM.03784-12

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911-917. https://doi.org/10.1139/o59-099

Brühl, L. (2014). Fatty acid alterations in oils and fats during heating and frying. *European Journal of Lipid Science and Technology*, *116*(6), 707-715. https://doi.org/10.1002/ejlt.201300273

Collins, Y. F., McSweeney, P. L., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal*, *13*(11), 841-866. https://doi.org/10.1016/S0958-6946(03)00109-2

Di Cagno, R., Filannino, P., & Gobbetti, M. (2017). Lactic acid fermentation drives the optimal volatile flavor-aroma profile of pomegranate juice. *International Journal of Food Microbiology*, *248*, 56-62. https://doi.org/10.1016/j.ijfoodmicro.2017.02.014

Di Cagno, R., Filannino, P., Cantatore, V., & Gobbetti, M. (2019). Novel solid-state fermentation of bee-collected pollen emulating the natural fermentation process of bee bread. *Food Microbiology*, *82*, 218-230. https://doi.org/10.1016/j.fm.2019.02.007

Di Cagno, R., Filannino, P., Vincentini, O., Lanera, A., Cavoski, I., & Gobbetti, M. (2016). Exploitation of *Leuconostoc mesenteroides* strains to improve shelf life, rheological, sensory and functional features of prickly pear (*Opuntia ficus-indica* L.) fruit puree. *Food Microbiology*, *59*, 176-189. https://doi.org/10.1016/j.fm.2016.06.009

Di Cagno, R., Minervini, G., Rizzello, C. G., De Angelis, M., & Gobbetti, M. (2011). Effect of lactic acid fermentation on antioxidant, texture, color and sensory properties of red and green smoothies. *Food Microbiology*, *28*(5), 1062-1071. https://doi.org/10.1016/j.fm.2011.02.011

Filannino, P., Cavoski, I., Thlien, N., Vincentini, O., De Angelis, M., Silano, M., Gobbetti, M., & Di Cagno, R. (2016). Lactic acid fermentation of cactus cladodes (*Opuntia ficus-indica* L.) generates flavonoid derivatives with antioxidant and anti-inflammatory properties. *PLoS One*, 11(3), e0152575. https://doi.org/10.1371/journal.pone.0152575

Filannino, P., Di Cagno, R., & Gobbetti, M. (2018). Metabolic and functional paths of lactic acid bacteria in plant foods: get out of the labyrinth. *Current Opinion in Biotechnology*, 49, 64-72. https://doi.org/10.1016/j.copbio.2017.07.016

Filannino, P., Di Cagno, R., Trani, A., Cantatore, V., Gambacorta, G., & Gobbetti, M. (2017). Lactic acid fermentation enriches the profile of biogenic compounds and enhances the functional features of common purslane (*Portulaca oleracea* L.). *Journal of Functional Foods*, *39*, 175-185. https://doi.org/10.1016/j.jff.2017.10.022

Filannino, P., Tlais, A. Z., Morozova, K., Cavoski, I., Scampicchio, M., Gobbetti, M., & Di Cagno, R. (2020). Lactic acid fermentation enriches the profile of biogenic fatty acid derivatives of avocado fruit (*Persea americana* Mill.). *Food Chemistry*, *317*, 126384. https://doi.org/10.1016/j.foodchem.2020.126384

Fiorino, G. M., Losito, I., De Angelis, E., Arlorio, M., Logrieco, A. F., & Monaci, L. (2019). Assessing fish authenticity by direct analysis in real time-high resolution mass spectrometry and multivariate analysis: Discrimination between wild-type and farmed salmon. *Food Research International*, *116*, 1258-1265. https://doi.org/10.1016/j.foodres.2018.10.013

Fregapane, G., Ojeda-Amador, R. M., & Salvador, M. D. (2019). Virgin walnut (*Juglans regia* L.) oil. In *Fruit oils: Chemistry and Functionality* (pp. 133-147). Springer, Cham.

Fuka, M. M., Tanuwidjaja, I., Maksimovic, A. Z., Zunabovic-Pichler, M., Kublik, S., Hulak, N., Domig, K. J., & Schloter, M. (2020). Bacterial diversity of naturally fermented game meat sausages: Sources of new starter cultures. *Lwt*, *118*, 108782. https://doi.org/10.1016/j.lwt.2019.108782

Gangopadhyay, N. (2021). Oils Extracted from Nuts and Grains. *Oil and Oilseed Processing: Opportunities and Challenges*, 61-79. https://doi.org/10.1002/9781119575313.ch4

Guasch-Ferré, M., Li, J., Hu, F. B., Salas-Salvadó, J., & Tobias, D. K. (2018). Effects of walnut consumption on blood lipids and other cardiovascular risk factors: an updated meta-analysis and systematic review of controlled trials. *The American Journal of Clinical Nutrition*, 108(1), 174-187. https://doi.org/10.1093/ajcn/nqy091

Hira, T., Ogasawara, S., Yahagi, A., Kamachi, M., Li, J., Nishimura, S., Sakaino, M., Yamashita, T., Kishino, S., Ogawa, J., & Hara, H. (2018). Novel mechanism of fatty acid sensing in enteroendocrine cells: specific structures in oxo-fatty acids produced by gut bacteria are responsible for CCK Secretion in STC-1 cells via GPR40. *Molecular Nutrition & Food Research*, 62(19), 1800146. https://doi.org/10.1002/mnfr.201800146

771 Hosomi, K., Kiyono, H., Kunisawa, J. (2020). Fatty acid metabolism in the host and commensal
772 bacteria for the control of intestinal immune responses and diseases. *Gut Microbes, 11*(3),
773 276-284. https://doi.org/10.1080/19490976.2019.1612662
774 Jang, E. J., Choi, W. R., Kim, S. Y., Hong, S. S., Rhee, I., Lee, S. J., Choi, S. W., Choi, H. G., &
775 Lim, S. J. (2017). 2-Hydroxyoleic acid-inserted liposomes as a multifunctional carrier of
776 anticancer drugs. *Drug Delivery*, 24(1), 1587-1597.
777 https://doi.org/10.1080/10717544.2017.1388452
778 Kellerer, T., Kleigrewe, K., Brandl, B., Hofmann, T., Hauner, H., & Skurk, T. (2021). Fatty acid
779 esters of hydroxy fatty acids (FAHFAs) are associated with diet, BMI, and age. *Frontiers*
780 *in Nutrition, 8*, 436. https://doi.org/10.3389/fnut.2021.691401
781 Kim, K. R., & Oh, D. K. (2013). Production of hydroxy fatty acids by microbial fatty acid-
782 hydroxylation enzymes. *Biotechnology Advances, 31*(8), 1473-1485.
783 https://doi.org/10.1016/j.biotechadv.2013.07.004
784 Kodani, S. D., & Morisseau, C. (2019). Role of epoxy-fatty acids and epoxide hydrolases in the
785 pathology of neuro-inflammation. *Biochimie, 159*, 59-65.
786 https://doi.org/10.1016/j.biochi.2019.01.020
787 Li, J., Huang, Q., Zheng, X., Ge, Z., Lin, K., Zhang, D., Chen, Y., Wang, B., & Shi, X. (2020).
788 Investigation of the lactic acid bacteria in kazak cheese and their contributions to cheese
789 fermentation. *Frontiers in Microbiology, 11*, 228.
790 https://doi.org/10.3389/fmicb.2020.00228
791 Liang, N., Tang, K., Curtis, J. M., & Gänzle, M. G. (2020). Identification and quantitation of
792 hydroxy fatty acids in fermented sausage samples. *Journal of Agricultural and Food*
793 *Chemistry*, 68(32), 8648-8657. https://doi.org/10.1021/acs.jafc.0c02688

Liu, W., Pu, X., Sun, J., Shi, X., Cheng, W., & Wang, B. (2022). Effect of *Lactobacillus plantarum* on functional characteristics and flavor profile of fermented walnut milk. *LWT*, 160, 113254. https://doi.org/10.1016/j.lwt.2022.113254

Losito, I., Facchini, L., Valentini, A., Cataldi, T. R., & Palmisano, F. (2018). Fatty acidomics: Evaluation of the effects of thermal treatments on commercial mussels through an extended characterization of their free fatty acids by liquid chromatography–Fourier transform mass spectrometry. *Food Chemistry*, *255*, 309-322. https://doi.org/10.1016/j.foodchem.2018.02.073

Mao, B., Guo, W., Huang, Z., Tang, X., Zhang, Q., Yang, B., Zhao, J., Cui, S., Zhang, H. (2022). Production of conjugated fatty acids in probiotic-fermented walnut milk with the addition of lipase. *LWT*, 172, 114204. https://doi.org/10.1016/j.lwt.2022.114204

McSweeney, P. L., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait*, *80*(3), 293-324. https://doi.org/10.1051/lait:2000127

Nagatake, T., Kunisawa, J. (2019). Emerging roles of metabolites of ω3 and ω6 essential fatty acids in the control of intestinal inflammation. *International Immunology*, *31*(9), 569-577. https://doi.org/10.1093/intimm/dxy086

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., & Thonart, P. (2011). Antifungal activity of 2 lactic acid bacteria of the Weissella genus isolated from food. *Journal of Food Science*, *76*(6), M305-M311. https://doi.org/10.1111/j.1750-3841.2011.02257.x

Paluchova, V., Vik, A., Cajka, T., Brezinova, M., Brejchova, K., Bugajev, V., Draberova, L., Draber, P., Buresova, J., Kroupova, P., Bardova, K., Rossmeisl, M., Kopecky, J., Hansen, T. V., Kuda, O. (2020). Triacylglycerol-rich oils of marine origin are optimal nutrients for

induction of polyunsaturated docosahexaenoic acid ester of hydroxy linoleic acid (13-DHAHLA) with anti-inflammatory properties in mice. *Molecular Nutrition & Food Research*, *64*(11), 1901238. https://doi.org/10.1002/mnfr.201901238

Pardatscher, R., & Schweigkofler, W. (2009). Microbial biodiversity associated with walnut *Juglans regia* L. in south Tyrol (Italy). *Mitt Klosterneuburg*, *59*, 17-23.

Porter, N.A., Caldwell, S.E., & Mills, K.A. (1995). Mechanisms of Free Radical Oxidation of Unsaturated Lipids. *Lipids*, *30*(4), 277-290. https://doi.org/10.1007/BF02536034

Pujo, J., Petitfils, C., Le Faouder, P., Eeckhaut, V., Payros, G., Maurel, S., Perez-Berezo, T., Van Hul, M., Barreau, F., Blanpied, C., Chavanas, S., Van Immerseel, F., Bertrand-Michel, J., Oswald, E., Knauf, C., Dietrich, G., Cani, P. D., Cenac, N. (2021). Bacteria-derived long chain fatty acid exhibits anti-inflammatory properties in colitis. *Gut*, *70*(6), 1088-1097. http://dx.doi.org/10.1136/gutjnl-2020-321173

Qin, X., Li, P., Lu, S., Sun, Y., Meng, L., Hao, J., & Fan, S. (2020). Phosphoproteomic analysis of lettuce (*Lactuca sativa* L.) reveals starch and sucrose metabolism functions during bolting induced by high temperature. *Plos One*, *15*(12), e0244198. https://doi.org/10.1371/journal.pone.0244198

Rocchetti, G., Rebecchi, A., Dallolio, M., Braceschi, G., Domínguez, R., Dallolio, G., Trevisan, M., Lorenzo, J.M., Lucini, L. (2021). Changes in the chemical and sensory profile of ripened Italian salami following the addition of different microbial starters. *Meat Science*, 180, 108584. https://doi.org/10.1016/j.meatsci.2021.108584

Saini, R. K., & Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance—A review. *Life Sciences*, *203*, 255-267. https://doi.org/10.1016/j.lfs.2018.04.049

840 Schoeler, M., Caesar, R. (2019). Dietary lipids, gut microbiota and lipid metabolism. *Reviews in*
841 *Endocrine and Metabolic Disorders*, *20*(4), 461-472. https://doi.org/10.1007/s11154-019-
842 09512-0
843 Takeuchi, M., Kishino, S., Park, S. B., Hirata, A., Kitamura, N., Saika, A., & Ogawa, J. (2016).
844 Efficient enzymatic production of hydroxy fatty acids by linoleic acid Δ9 hydratase from
845 *Lactobacillus plantarum* AKU 1009a. *Journal of Applied Microbiology*, *120*(5), 1282-
846 1288. https://doi.org/10.1111/jam.13088
847 Tlais, A. Z. A., Kanwal, S., Filannino, P., Albiac, M. A., Gobbetti, M., & Di Cagno, R. (2022).
848 Effect of sequential or ternary starters-assisted fermentation on the phenolic and
849 glucosinolate profiles of sauerkraut in comparison with spontaneous fermentation. *Food*
850 *Research International*, *156*, 111116. DOI: 10.1016/j.foodres.2022.111116
851 Tlais, A. Z., Da Ros, A., Filannino, P., Vincentini, O., Gobbetti, M., & Di Cagno, R. (2021).
852 Biotechnological re-cycling of apple by-products: A reservoir model to produce a dietary
853 supplement fortified with biogenic phenolic compounds. *Food Chemistry*, *336*, 127616.
854 https://doi.org/10.1016/j.foodchem.2020.127616
855 van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L., & van Geel-Schutten, I. G. (2006).
856 Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic
857 acid bacteria. *Microbiology and Molecular Biology Reviews*, *70*(1), 157-176.
858 https://doi.org/10.1128/MMBR.70.1.157-176.2006
859 Yang, B., Chen, H., Stanton, C., Ross, R. P., Zhang, H., Chen, Y. Q., & Chen, W. (2015). Review
860 of the roles of conjugated linoleic acid in health and disease. *Journal of Functional*
861 *Foods*, *15*, 314-325. https://doi.org/10.1016/j.jff.2015.03.050

Yang, X., Hu, W., Xiu, Z., Jiang, A., Yang, X., Ji, Y., Guan, Y., & Feng, K. (2020). Microbial dynamics and volatilome profiles during the fermentation of Chinese northeast sauerkraut by *Leuconostoc mesenteroides* ORC 2 and *Lactobacillus plantarum* HBUAS 51041 under different salt concentrations. *Food Research International*, *130*, 108926. https://doi.org/10.1016/j.foodres.2019.108926

Zhao, W., Liu, M., Qin, Y., Bing, H., Zhang, F., & Zhao, G. (2022). Characterization and functional of four mutants of hydroxy fatty acid dehydrogenase from *Lactobacillus plantarum* p-8. *FEMS Microbiology Letters*. https://doi.org/10.1093/femsle/fnac060

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

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

886

887

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 1

Click here to access/download;Figure(s);Figure 1.tif

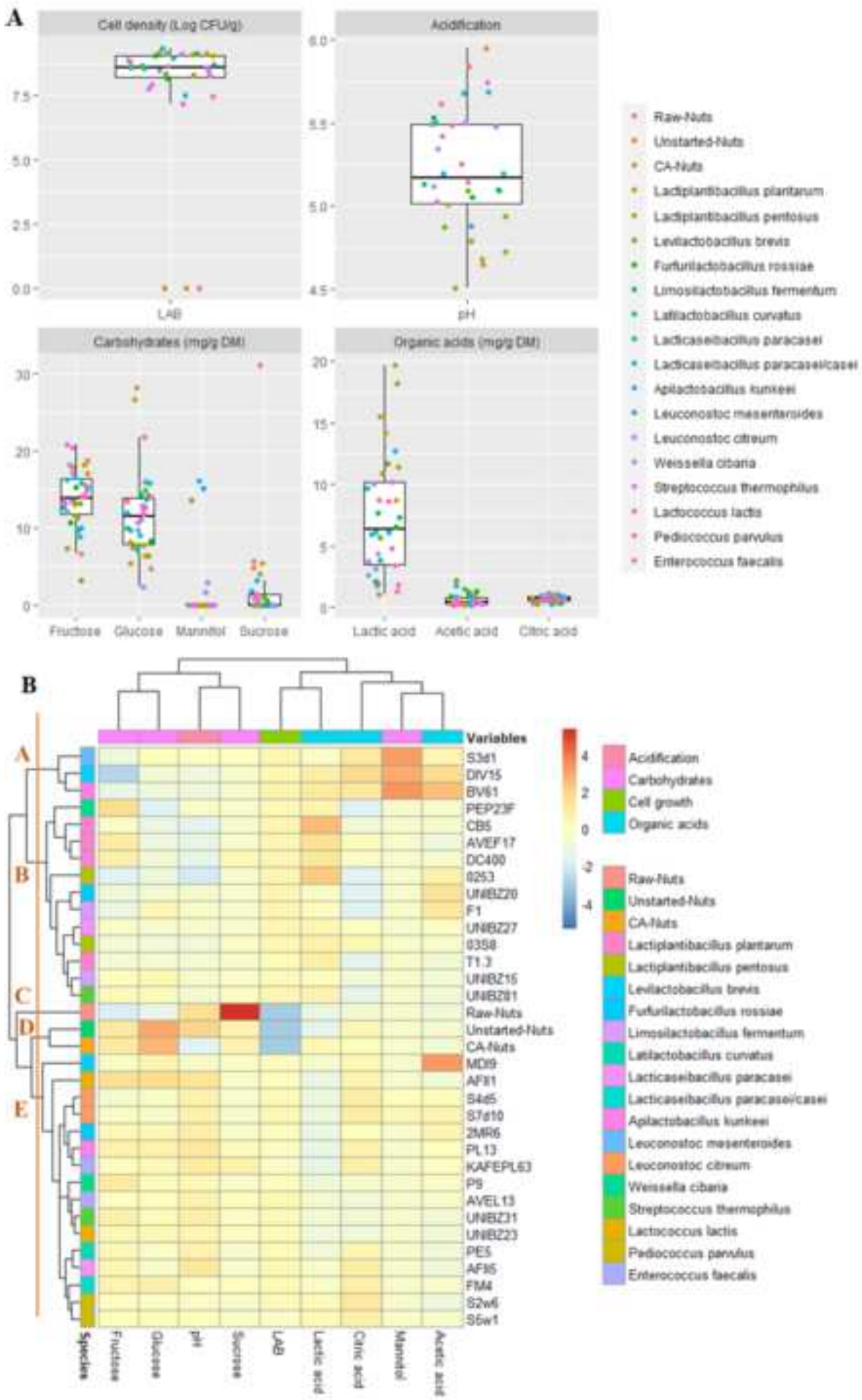


Figure 2

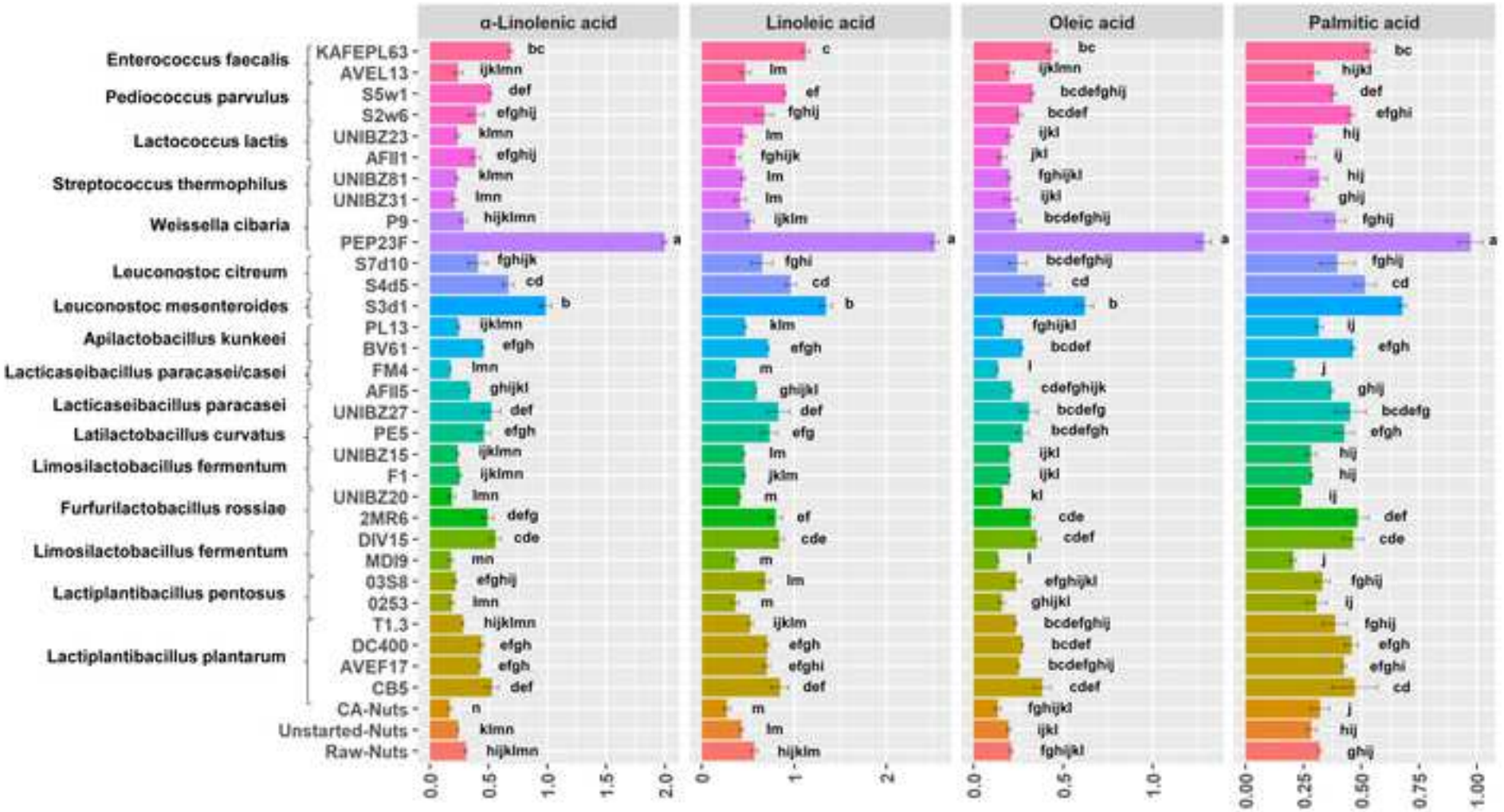


Figure 3

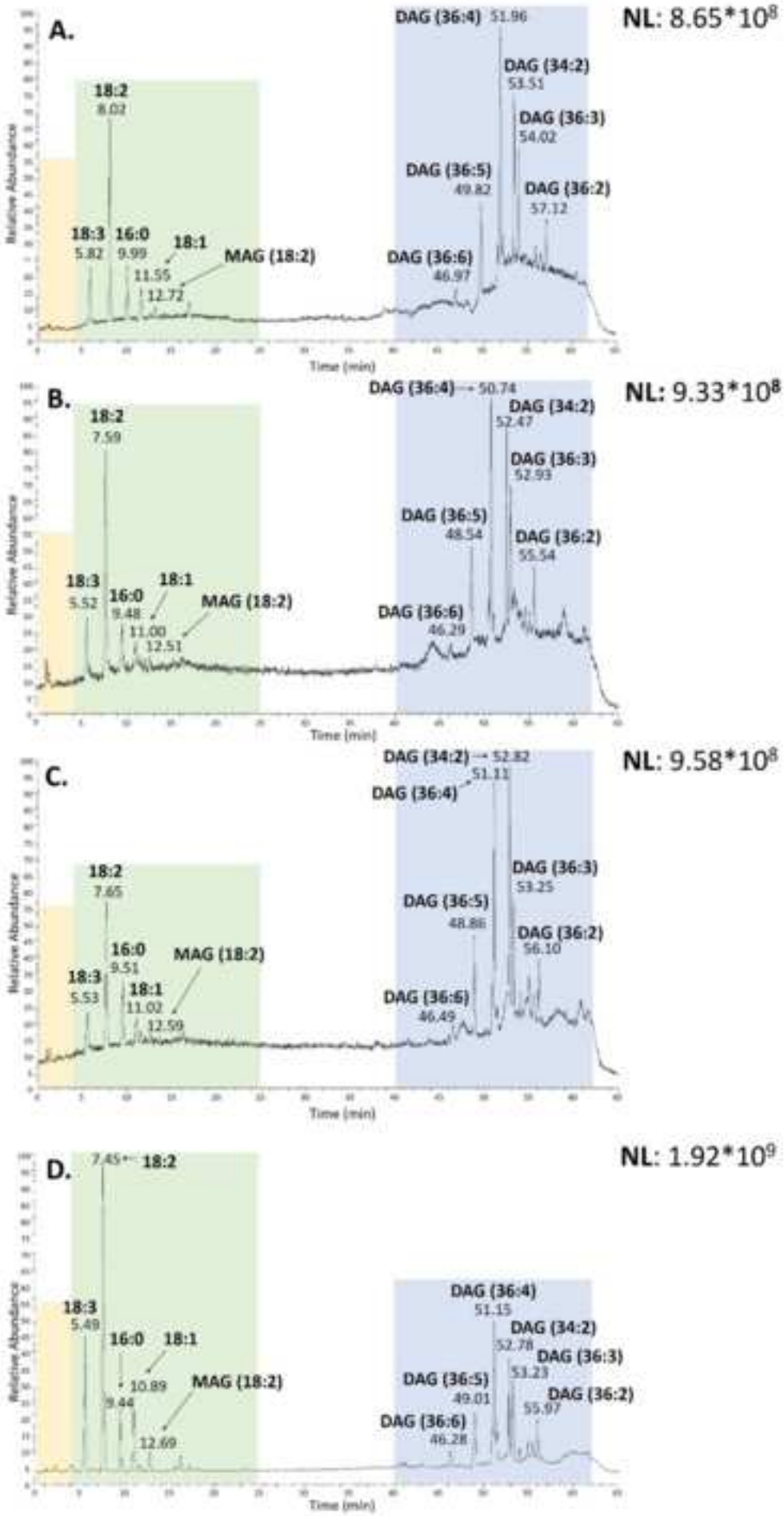


Figure 4

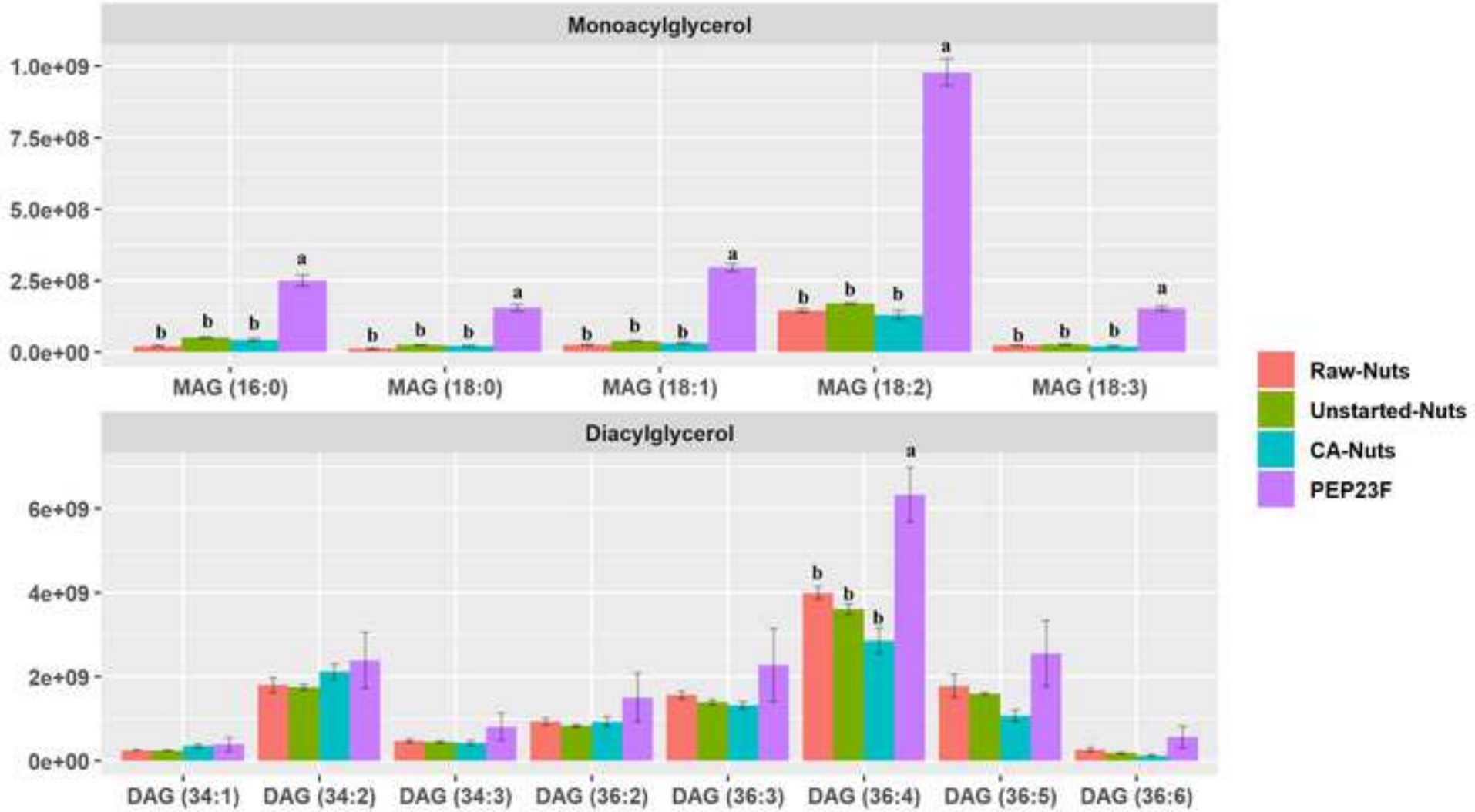
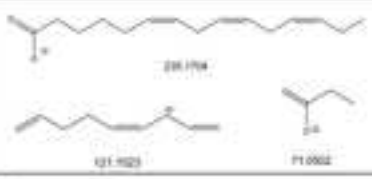
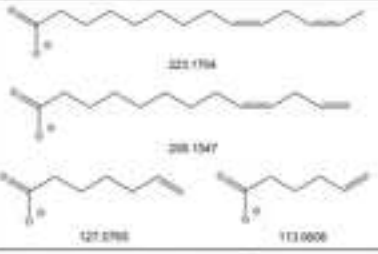
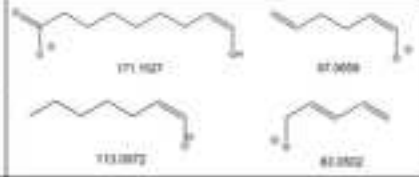
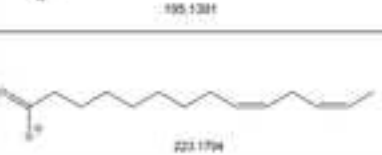
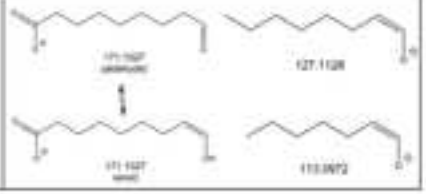


Figure 5

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)	
	17-hydroxy-α-linolenic acid (17-hydroxy-octadeca-9,12,15-trienoic acid)	
	9-hydroxy-octadeca-10,12,15-trienoic acid	
295.2279	13-hydroxy-octadeca-9,11-dienoic acid	
	15-hydroxy-linoleic acid (15-hydroxy-octadeca-9,12-dienoic acid)	
	9,10- epoxy-octadeca-12-enoic acid	

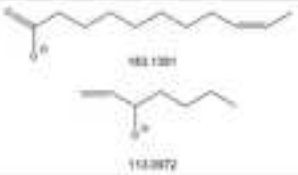
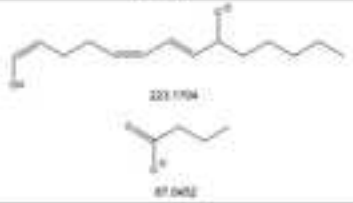
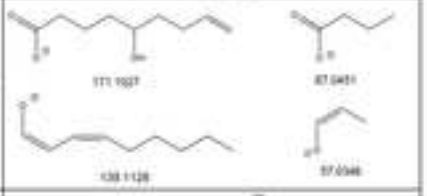
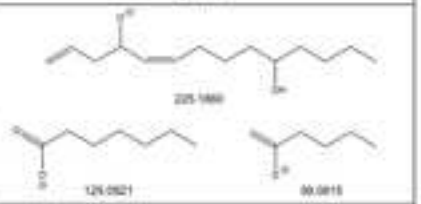
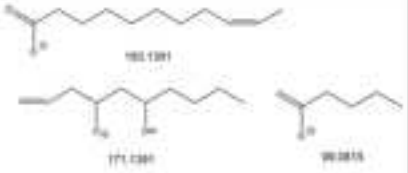
Exact m/z	Identified compound	Diagnostic product ions detected in MS/MS spectra
297.2435	14-hydroxy-oleic acid (14-hydroxy-octadeca-9-enoic acid)	
311.2228	5,13-dihydroxy-octadeca-9,11-dienoic acid	
	5,10-dihydroxy-octadeca-8,12-dienoic acid	
	10,16-hydroxy-octadeca-8,12-dienoic acid	
313.2384	8,14-dihydroxy-oleic acid (8,14-dihydroxy-octadeca-9-enoic acid)	
	12,14-dihydroxy-oleic acid (12,14-dihydroxy-octadeca-9-enoic acid)	

Figure 6

Click here to access/download;Figure(s);Figure 6.tif

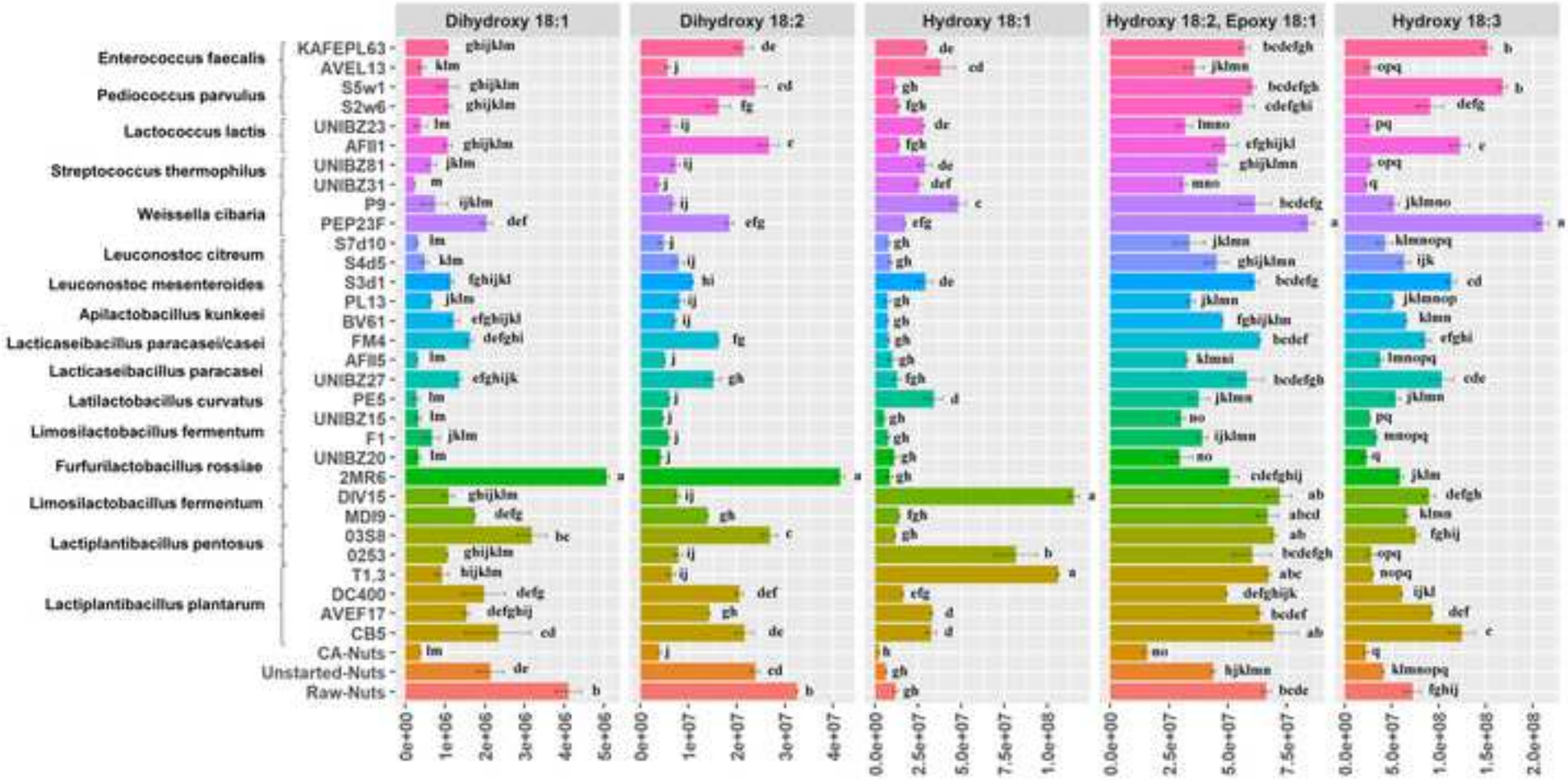


Figure S1. Principal component analysis (PCA) biplot, based on free fatty acids concentration (mg g^{-1} DM) of Raw-Nuts, Unstarted-Nuts, CA-Nuts and Fermented-Nuts (B).

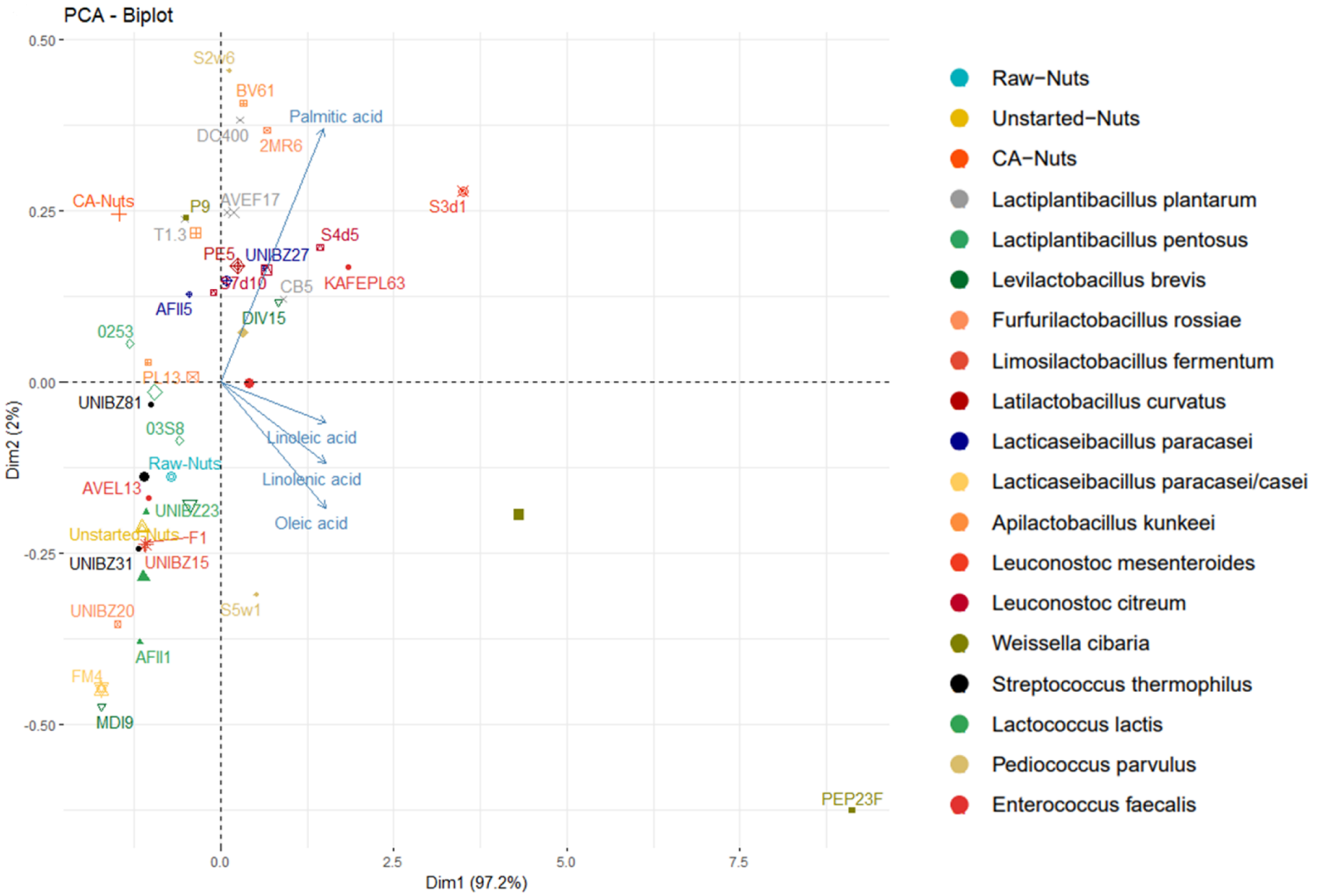


Figure S2. Principal component analysis (PCA) biplot, based on hydroxy/epoxy fatty acids concentration (Unit) of Raw-Nuts, Unstarted-Nuts, CA-Nuts and Fermented-Nuts (B).

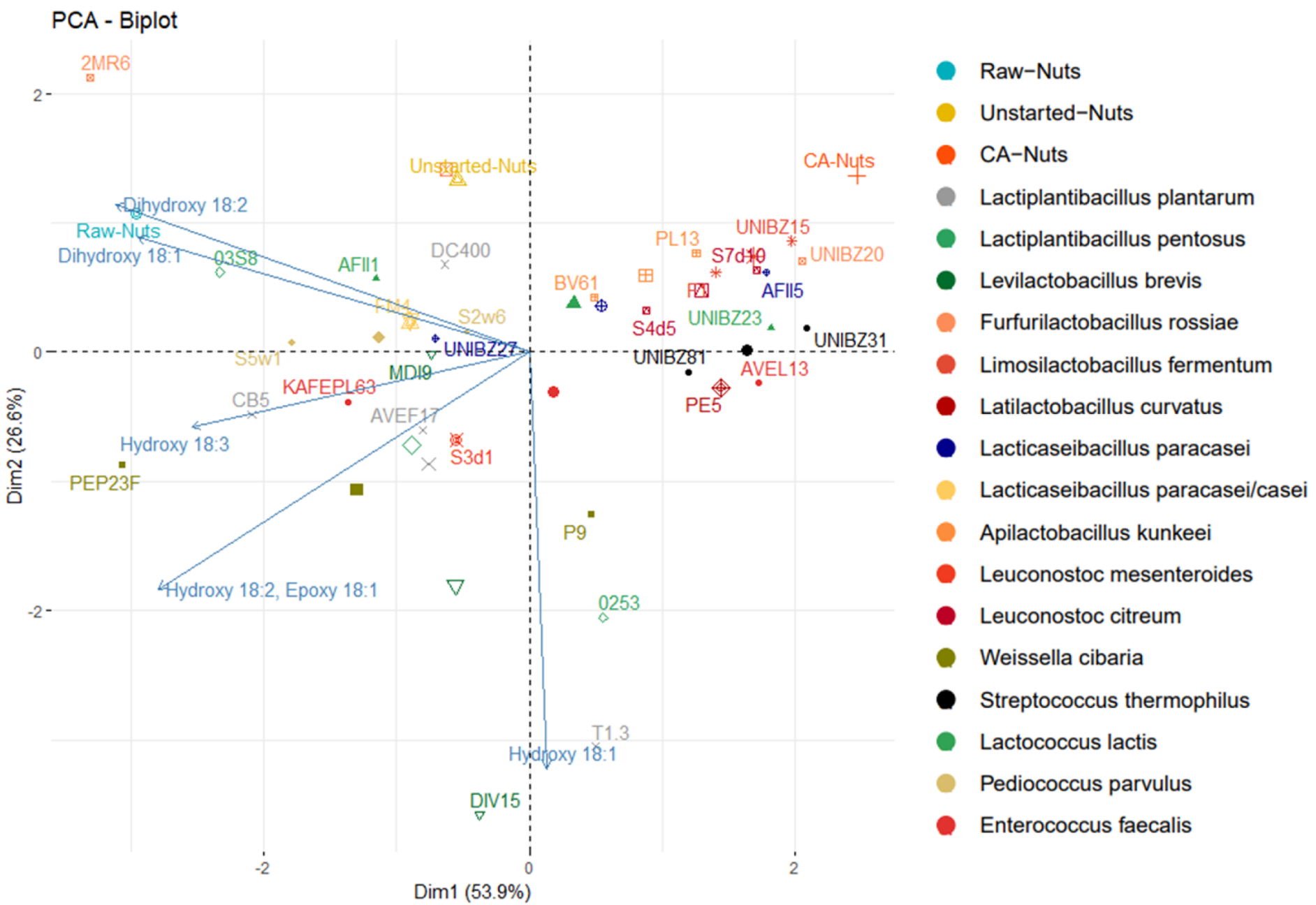


Table S1. Cell density (Log CFU g^{-1}) of lactic acid bacteria, pH, and concentration (mg g^{-1} DM) of carbohydrates and organic acids of raw walnuts (Raw-Nuts), walnuts without microbial inoculum (Unstarted-Nuts), walnuts without microbial inoculum and chemically acidified with lactic acid (CA-Nuts)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

	Cell density (Log CFU g^{-1})	pH	Carbohydrates (mg g^{-1} DM)				Organic acids (mg g^{-1} DM)		
Samples			**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}
***Lactiplantibacillus plantarum* 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}
***Lacti. plantarum* T1.3**	8.27 ± 0.06^{cdefg}	5.01 ± 0.04^{op}	11.37 ± 1.56^{hijkl}	8.27 ± 0.10^{jklmno}	-	-	$11.38 \pm 0.05f^{g}$	0.34 ± 0.00^{qp}	0.24 ± 0.00^{fg}
***Lacti. plantarum* CB5**	9.02 ± 0.01^{abc}	4.68 ± 0.08^{t}	$13.15 \pm 0.27^{defghijk}$	5.44 ± 0.3^{1mno}	-	-	19.66 ± 0.02^{a}	0.53 ± 0.00^{m}	0.41 ± 0.00^{fg}
***Lacti. plantarum* 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}
***Lactiplantibacillus pentosus* 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^{ji}	0.39 ± 0.01^{fg}
***Lacti. pentosus* 0253**	9.14 ± 0.11^{ab}	4.51 ± 0.01^{u}	7.47 ± 1.33^{klm}	6.49 ± 1.4^{1mno}	-	-	18.19 ± 0.05^{b}	0.28 ± 0.00^{sr}	0.97 ± 0.91^{cdef}
***Levilactobacillus brevis* MDI9**	8.31 ± 0.21^{bcdef}	5.09 ± 0.01^{lmn}	$13.21 \pm 0.58^{defghijk}$	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}
***Lev. brevis* 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}
***Furfurilactobacillus rossiae* 2MR6**	8.16 ± 0.09^{defgh}	5.53 ± 0.03^{e}	$15.30 \pm 0.32^{abcdefgh}$	14.20 ± 0.00^{abcdef}	-	-	2.06 ± 0.00^{rs}	0.72 ± 0.00^{lk}	0.87 ± 0.03^{fg}
***F. rossiae* UNIBZ20**	9.03 ± 0.18^{abc}	5.05 ± 0.03^{mno}	10.72 ± 0.39^{ijkl}	8.33 ± 0.57^{jklmno}	-	-	6.57 ± 0.11^{j}	0.32 ± 0.00^{rq}	1.34 ± 0.06^{dcb}
***Limosilactobacillus fermentum* 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}
***Lim. fermentum* UNIBZ15**	8.53 ± 0.14^{abcde}	5.09 ± 0.03^{lmn}	$13.60 \pm 0.16^{cdefghij}$	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}
***Latilactobacillus curvatus* PE5**	8.56 ± 0.04^{abcde}	5.50 ± 0.01^{ef}	$15.27 \pm 0.23^{abcdefgh}$	$10.09 \pm 0.72^{fghijklm}$	-	-	3.86 ± 0.24^{no}	0.83 ± 0.00^{gi}	0.27 ± 0.01^{fg}
***Lacticaseibacillus paracasei* 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}
***L. paracasei* 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}
***L. paracasei/casei* 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}
***Apilactobacillus kunkeei* 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}
***A. kunkeei* 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}
***Leuconostoc mesenteroides* S3d1**	8.95 ± 0.02^{abc}	5.20 ± 0.04^{ijk}	10.07 ± 0.01^{jklm}	$11.93 \pm 1.18^{defghijk}$	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}
***Leuconostoc citreum* S4d5**	8.45 ± 0.05^{abcde}	5.49 ± 0.08^{fg}	12.14 ± 0.62^{fghijkl}	$11.14 \pm 0.19^{efghijkl}$	1.71 ± 0.12^{bc}	-	3.11 ± 0.01^{pq}	0.95 ± 0.00^{de}	0.65 ± 0.16^{defg}
***Leuc. citreum* S7d10**	8.88 ± 0.02^{abcd}	5.48 ± 0.04^{efg}	$13.26 \pm 0.53^{defghijk}$	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}
***Weissella cibaria* 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}	$0.40 \pm 0.00f^{g}$
***W. cibaria* P9**	7.74 ± 0.15^{fghi}	5.34 ± 0.01^{h}	18.27 ± 0.77^{abcd}	$12.98 \pm 3.11^{bcdefghij}$	-	0.50 ± 0.32^{gfe}	3.75 ± 0.65^{nop}	0.61 ± 0.00^{n}	0.40 ± 0.01^{fg}
***Streptococcus thermophilus* UNIBZ31**	8.57 ± 0.16^{abcde}	5.50 ± 0.04^{efg}	17.21 ± 0.72^{abcde}	$12.88 \pm 0.48^{bcdefghij}$	-	0.91 ± 0.12^{gfe}	4.39 ± 0.05^{mn}	0.52 ± 0.00^{om}	0.27 ± 0.00^{fg}
***S. thermophilus* UNIBZ81**	9.11 ± 0.17^{ab}	5.03 ± 0.03^{no}	$14.78 \pm 0.13^{bcdefghi}$	$12.55 \pm 0.55^{cdefghij}$	-	0.82 ± 0.24^{gfe}	10.19 ± 0.12^{h}	0.37 ± 0.01^{p}	0.23 ± 0.02^{fg}
***Lactococcus lactis* 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}
***Lc. lactis* UNIBZ23**	8.27 ± 0.06^{cdefg}	5.42 ± 0.01^{g}	$15.53 \pm 0.39^{abcdefgh}$	$14.0\ 7 \pm 0.32^{abcdef}$	-	1.40 ± 0.04^{gfe}	3.43 ± 0.05^{op}	0.48 ± 0.01^{o}	0.18 ± 0.00^{g}
***Pediococcus parvulus* S2w6**	8.59 ± 0.09^{abcde}	5.15 ± 0.01^{jkl}	$13.49 \pm 0.33^{defghijk}$	10.78 ± 0.42^{fghijkl}	-	-	8.65 ± 0.01^{i}	1.01 ± 0.00^{b}	0.14 ± 0.01^{g}
***P. parvulus* S5w1**	7.15 ± 0.2^{i}	5.25 ± 0.03^{i}	14.25 ± 1.88^{cdefghi}	$12.50 \pm 2.41^{cdefghij}$	-	0.05 ± 0.07^{gf}	8.71 ± 0.02^{i}	0.90 ± 0.00^{f}	0.42 ± 0.01^{fg}
***Enterococcus faecalis* AVEL13**	8.76 ± 0.06^{abcd}	5.49 ± 0.05^{efg}	$13.66 \pm 2.49^{cdefghij}$	$11.90 \pm 1.18^{defghijk}$	-	1.34 ± 0.80^{gfe}	4.77 ± 0.05^{m}	0.48 ± 0.01^{o}	0.41 ± 0.04^{fg}
***E. faecalis* KAFEPL63**	7.45 ± 0.02^{hi}	5.62 ± 0.01^{d}	14.08 ± 0.50^{cdefghi}	$13.50 \pm 0.60^{abcdefgh}$	-	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}
***Enterococcus faecalis* AVEL13**	0.47 ± 0.05^{lm}	0.24 ± 0.04^{ijklmn}	0.20 ± 0.02^{ijklmn}	0.30 ± 0.02^{hijkl}
***E. faecalis* KAFEPL63**	1.13 ± 0.04^{c}	0.69 ± 0.02^{bc}	0.44 ± 0.03^{bc}	0.54 ± 0.02^{bc}
***Levilactobacillus brevis* DIV15**	0.84 ± 0.05^{cde}	0.56 ± 0.05^{cde}	0.35 ± 0.03^{cdef}	0.47 ± 0.04^{cde}
***L. brevis* MDI9**	0.36 ± 0.02^{m}	0.18 ± 0.02^{mn}	0.14 ± 0.00^{l}	0.21 ± 0.01^{j}
***Latilactobacillus curvatus* PE5**	0.74 ± 0.09^{efg}	0.46 ± 0.06^{efgh}	0.27 ± 0.04^{bcdefgh}	0.43 ± 0.04^{efgh}
***Limosilactobacillus fermentum* UNIBZ15**	0.46 ± 0.01^{lm}	0.24 ± 0.01^{jklmn}	0.20 ± 0.01^{ijkl}	0.28 ± 0.03^{hij}
***L. fermentum* F1**	0.47 ± 0.01^{mjklm}	0.25 ± 0.02^{ijklmn}	0.20 ± 0.01^{ijkl}	0.29 ± 0.00^{hij}
***Apilactobacillus kunkeei* BV61**	0.72 ± 0.01^{efgh}	0.45 ± 0.00^{efgh}	0.27 ± 0.01^{bcdef}	0.46 ± 0.01^{efgh}
***Apilactobacillus kunkeei* PL13**	0.47 ± 0.02^{klm}	0.25 ± 0.01^{ijklmn}	0.16 ± 0.00^{fghijkl}	0.32 ± 0.02^{ij}
***Lacticaseibacillus paracasei* UNIBZ27**	0.83 ± 0.12^{def}	0.52 ± 0.08^{def}	0.31 ± 0.05^{bcdefg}	0.45 ± 0.07^{bcdefg}
***L. paracasei* AFII5**	0.59 ± 0.00^{ghijkl}	0.34 ± 0.00^{ghijkl}	$0.21 \pm 0.01^{cdefghijk}$	0.37 ± 0.01^{ghij}
***L. paracasei/casei* FM4**	0.37 ± 0.00^{m}	0.18 ± 0.00^{lmn}	0.13 ± 0.00^{l}	0.21 ± 0.01^{j}
***Lactiplantibacillus pentosus* 0253**	0.37 ± 0.04^{m}	0.19 ± 0.02^{lmn}	0.16 ± 0.02^{ghijkl}	0.31 ± 0.05^{ij}
***L. pentosus* 03-S-8**	0.69 ± 0.06^{lm}	0.22 ± 0.01^{efghij}	0.24 ± 0.03^{efghijkl}	0.33 ± 0.03^{fghij}
***Lactiplantibacillus plantarum* CB5**	0.85 ± 0.10^{def}	0.53 ± 0.06^{def}	0.38 ± 0.05^{cdef}	0.47 ± 0.10^{cd}

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

Credit Author 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 interests

x 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: