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Revealing the bacterial abundance and diversity in brines from started Spanish-style green table olives

D. Correa-Galeote [a,b,*], I. Ghomari [c], A. Asehraou [c], J. González-López [a,b]

[a] University of Granada. Faculty of Pharmacy. Department of Microbiology, Granada, Spain
[b] University of Granada. Water Research Institute, Granada, Spain
[c] University Mohammed Premier. Faculty of Sciences. Department of Biology, Oujda, Morocco

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ABSTRACT

The influence of the inoculum on bacterial communities of Spanish-style green table olive brinesis very limited. This work assessed the size and structure of the bacterial communities in olive brines inoculated with *Lactiplantibacillus plantarum*. Also, a new qPCR assay was developed to determine the specific abundance of this genus. Absolute abundances of total *Bacteria* and *L. plantarum* decrease steadily during the fermentation. Similarly, there were differences in both populations according to the container origin. On the other hand, the dominant bacterial genera were (in decreasing abundance): *Vibrio*, *Marinilactibacillus*, *Lactiplantibacillus*, *Enterococcus*, *Secundilactobacillus*, *Loigolactobacillus*, *Amphibacillus*, *Pediococcus*, *Alkalibacterium*, *Halolactibacillus*, *Weissella*, *Lentilactobacillus*, and *Paucilactobacillus*. Colonisation and proliferation of several different genera within the *Lactobacillacea* family was allowed despite the use of a starter. Bacterial structure presented a broad intra-specific diversity among the different brines. Also, it was revealed that NaCl concentration was modulated the size and structure of the bacterial communities.

1. Introduction

Fermented foods are nourishment products manufactured through enzymatic conversions of major and minor food components by microbial organisms. In recent years, fermented foods have gained popularity, increasing their consumption (German et al., 2009). Among these foods, fermented olives are of interest as their consumption is expanded year by year up to more than three million tons (IOC, 2020). Fermented olives are the most widespread fermented food in the Mediterranean area (Vaccalluzzo et al., 2020). Due to their high nutritional value and high content of bioactive compounds, mainly dietary fibre, antioxidants, and vitamins, they are regarded as functional food (Perpetuini et al., 2020; Vaccalluzzo et al., 2020). Spain stands out for its weight in world production, accounting for approximately 60% of the world production (Lucena-Padrós & Ruiz-Barba, 2019; Rodríguez-Gómez et al., 2014).

The fermentation of fruits of *Olea europaea* (mainly Gordal, Hojiblanca, and Manzanilla varieties) is carried out by lactic acid bacteria (LAB) spontaneously by adventitious microbiota or directed by using a starter (IOC, 2020). LAB have potential benefits on health and are considered a probiotic source, with a capacity to improve digestion of lactose, increase food digestibility, hypercholesterolemia reduction, and prevent intestinal infections (Campana et al., 2017; Champagne et al., 2005). Among the different LAB, many strains within the family *Lactobacillaceae* are prominent in traditional olive fermentations (Leal-Sánchez et al., 2003; Medina et al., 2018). In this sense, *Lactiplantibacillus plantarum* (Zheng et al., 2020) is one of the most often microorganisms isolated from fermented products, sourdough, fermented vegetables, and meats (Essid et al., 2009; Jung et al., 2019), coming dominant in spontaneous fermented olives (Blana et al., 2014; Kaltsa et al., 2015). Besides, *L. plantarum* is the most frequent microorganism in commercial starters for olive fermentation (Cosmai et al., 2018; De Angelis et al., 2015). *Lactiplantibacillus* is exploited to increase the organoleptic quality of food products (Sabatini et al., 2008) and prevent spoiling microorganisms growth (Benítez-Cabello et al., 2020). Additionally, it carries relevant properties to human health, as are vitamin and bacteriocin production, probiotic capacities, and potential anticancer agents (Evanovich et al., 2019; Li et al., 2016).

New molecular tools have revolutionised microbial food ecology as valuable alternatives to culture-dependent methods (Achilleos & Berthier, 2013). In this sense, recently, the genus *Lactobacillus* was emended based on whole-genome sequencing, resulting in 25 different genera, including the genus *Lactobacillus*, *Paralactobacillus*, and 23 novel

* Corresponding author. University of Granada. Faculty of Pharmacy. Department of Microbiology, Granada, Spain.
E-mail address: dcorrea@ugr.es (D. Correa-Galeote).

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genera (Zheng et al., 2020). Therefore, it is mandatory to re-evaluate the biodiversity of the LAB under this new perspective to provide better accuracy in the LAB diversity in different lactobacilli-enriched fermented foods. Furthermore, despite the great importance of *L. plantarum* in fermented foods, the scientific community lacks accurate molecular tools to assess specifically their total abundance. Different works have addressed the quantification of *L. plantarum* (Garg et al., 2009; Tsai et al., 2010; Zhang et al., 2012); however, the accuracy of these molecular tools to specifically amplify the *L. plantarum* populations is compromised.

On the other hand, Spain stands out for its weight in world production, accounting for approximately 60% of the world output (Rodríguez-Gómez et al., 2014). Few comprehensive scientific studies have evaluated bacterial communities' total abundance and structure of Spanish-style table green olive brines (Benítez-Cabello et al., 2020; Lucena-Padrós & Ruiz-Barba, 2019). However, the effect on olive brines' bacterial communities of the use of a starter is not well-established, and its control on non-desired microorganisms' growth is unknown. Furthermore, the intra-specific differences in each fermented container could drive at least a part of the microbial diversity developed specifically in each fermenter and different physicochemical factors can affect the bacterial communities in olive brines. Hence, their effect on the microbial needs to be evaluated, especially if the new *Lactobacillaceae* classification is considered.

For these purposes, quantifications of the total bacterial abundance and determination of the bacterial structure in brines retrieved from Spanish-style table green olives were made by qPCR and Illumina sequencing, respectively. Additionally, a qPCR assay for the specific quantification *L. plantarum* was developed. Finally, the effects of the abiotic variables (pH, titratable acidity, and NaCl concentrations) on the abundance and structure of the bacterial community were analysed. Therefore, the goal of this study was to address the dynamic of the size and structure of the bacterial diversity over the entire fermentation period determining the impact of sampling time and the use of different containers in bacterial communities in olives brines.

2. Material and methods

2.1. Origin of the samples and sampling strategy

Samples of fermented olives brines were taken in the 2019–2020 season from a manufacturing company in the province of Córdoba (South Spain) following a traditional Spanish-style protocol (revised by Ramírez et al., 2015). Briefly, fruits from the Hojiblanca variety in the ripening stage were supplied by local farmers to the manufacturing company in October. After that, fruits were subjected to a 6% NaOH solution for 6–8 h to reduce the bitter taste of oleuropein and other polyphenols, form organic acids that aid in subsequent fermentation, and permeabilising the fruit. Then, olives were washed several times with tap water to eliminate excess alkali. Subsequently, the fruits were placed in polyester vessels buried in the ground and located outdoors (called 'patios') and, posteriorly, covered with the brine solution (ca. 10% NaCl) for 180 days. Starter cultures of commercial *L. plantarum* (Bactoferm Vege-Start 100Chr, Hansen, Denmark) were added after seven days of fermentation.

Brine samples were collected under sterile conditions in triplicate from three independent vessels monthly. The brine samples were transported to the laboratory under refrigeration and then stored at −20 °C until use.

2.2. Physicochemical analyses

Analysis of pH was determined using a pH/Conductimeter (Consort C860, Thermo Fisher Scientific, USA) following the methodology proposed by Lucena-Padrós et al. (2014). Determination of titratable acidity and NaCl concentration were carried out using ion chromatography (861 Advanced Compact, Metrohm, Switzerland) as previously described by Lucena-Padrós et al. (2014) and Correa-Galeote et al. (2021), respectively. All measurements were made in triplicate.

2.3. DNA extraction

For DNA extractions, 50 mL from each brine sample was centrifuged (14,000 rpm, 30 min). The resulting biomasses were processed using the FastDNA-2 mL SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO, USA) according to Correa-Galeote et al. (2021). The quality and concentration of DNAs were determined using an Invitrogen Qubit 4 Fluorometer (Waltham, USA).

2.4. Quantitative polymerase chain reaction

Bacterial 16S rRNA gene quantitative PCR (qPCR) using primers 341F and 534R (Muyzer et al., 1993) was used as a proxy for the total *Bacteria* abundances. Amplification reactions and PCR conditions were following according to Correa-Galeote et al. (2013). To quantify the *L. plantarum* populations, a specific qPCR assay using primers planF (5′-CCGTTTATGCGGAACACCTAG-3′, Torriani et al., 2001), and planR (5′-TGCCCGCCGTACTTCCAAA-3′, this study) targeting the *recA* gene from *L. plantarum was made.* PCR reactions and the thermal profile conditions were the same as those used for the 16S rRNA gene quantification (except annealing temperature: 57 °C). All qPCR reactions were run on a QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA) by triplicate.

2.5. 16S rRNA gene high-throughput sequencing

DNAs were also subjected to bacterial 16S rRNA Illumina sequencing at the Institute of Parasitology and Biomedicine' López-Neyra (IPBLN-CSIC, Spain) using primers Pro341F/805R (Takahashi et al., 2014). Raw data from Illumina sequencing were processed using Mothur v1.44.1 software (Schloss et al., 2009). Default settings were used for merging, quality control, primer trimming, filtering, de-noising and chimeric filtering and conversion into unique sequences. The remaining unique sequences were clustered into operational taxonomic units (OTUs, 97% similarity), and OTUs with an abundance of only one sequence were removed. Finally, the taxonomical classification of the consensus sequence of each OTU was determined through the blast suite of the Geneious 2021.1.1 software (Biomatters, New Zealand) using the bacterial 16S rRNA NCBI database (https://www.ncbi.nlm.nih.gov/nuccore). The nucleotide sequences have been deposited in GeneBank under the accession numbers SUB9034252. Biodiversity indices were calculated according to Hill et al. (2003). A Tamura-Nei phylogenetic tree of the OTUs within the family *Lactobacillaceae* was constructed using Geneious.

2.6. Statistical analyses

Mean comparisons (Kruskal-Wallis test and Conover-Iman *post hoc* tests; $p < 0.05$) were determined in XLSTAT 2021.1.1 software (Addinsoft, USA), and Spearman's correlations analysis were made on the vegan R package. A co-occurrence correlation network was constructed through the Gephi v0.9.2 software (Gephi Consortium, France). The PC-ORD software v6.08 (MjM Software, USA) was used for nonmetric multidimensional scaling (NMS) analysis.

3. Results and discussion

3.1. Evolution of physicochemical characteristics through fermentation

The values of the pH, titratable acidity, and NaCl concentrations in the brines are shown in Table 1. No statistical differences were found for the pH values among the different vessels for the different fermentation

Table 1
Physico-chemical parameters analysed in the Spanish-style table green olive brines in three fermentation vessels. Lower-case letters indicate significant differences among sampling times for a given container, and capital letters indicate significant differences among containers for a given sampling time according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$), respectively.

		30 days	60 days	90 days	120 days	150 days	180 days
pH	Vessel A	4.48 ± 0.01 A a	4.28 ± 0.01 A b	4.25 ± 0.04 A b	4.16 ± 0.01 A c	4.10 ± 0.05 A cd	4.05 ± 0.02 A d
	Vessel B	4.48 ± 0.02 A a	4.31 ± 0.02 A b	4.26 ± 0.02 A c	4.16 ± 0.02 A d	4.09 ± 0.01 A e	4.02 ± 0.01 A f
	Vessel C	4.54 ± 0.03 A a	4.36 ± 0.02 A b	4.27 ± 0.03 A c	4.15 ± 0.01 A d	4.09 ± 0.02 A e	4.05 ± 0.01 A f
Acidity (g/100 mL)	Vessel A	0.85 ± 0.01 B d	1.01 ± 0.01 A c	1.01 ± 0.01 B c	1.09 ± 0.01 C b	1.11 ± 0.01 C b	1.13 ± 0.01 B a
	Vessel B	0.99 ± 0.01 A d	1.01 ± 0.01 A d	1.09 ± 0.01 A c	1.24 ± 0.01 A b	1.26 ± 0.01 A ab	1.28 ± 0.01 A a
	Vessel C	0.77 ± 0.01 C d	0.99 ± 0.01 A c	0.99 ± 0.01 C c	1.18 ± 0.01 B b	1.20 ± 0.01 B ab	1.22 ± 0.01 C a
[NaCl] (%)	Vessel A	8.22 ± 0.07 A d	8.29 ± 0.05 B cd	8.51 ± 0.07 A ab	8.41 ± 0.05 A abc	8.39 ± 0.07 C bcd	8.60 ± 0.06 B a
	Vessel B	8.12 ± 0.07 A bc	8.11 ± 0.05 C c	8.45 ± 0.04 A b	8.61 ± 0.06 A a	8.66 ± 0.04 B a	8.66 ± 0.03 B a
	Vessel C	8.50 ± 0.06 A b	8.84 ± 0.04 A a	8.77 ± 0.03 A b	8.76 ± 0.04 A b	8.83 ± 0.03 A a	8.84 ± 0.04 A a

times. However, the titratable acidity (expressed in grams of acid per 100 mL of brine) was statistically higher at vessel B than those of the other two containers. The salinity was significantly higher in vessel C at 60, 150, and 180 days. Regarding the effect of fermentation time, a significant reduction in pH was observed for the three containers over time. Inversely, a statistical increase in the acidity was found at the end of the fermentation. Fermentation time does not have an apparent effect on the NaCl concentration. Therefore, the specific physicochemical characteristics in each container and sampling time could drive at least a part of brine's microbial diversity.

The pH and acidity fell within the previous data reported for Spanish-style olive brines (Lucena-Padrós et al., 2014; Lucena-Padrós & Ruiz-Barba, 2019) and from other types of fermented olives Sicilian (Poiana & Romeo, 2006) and Morrocan (Abouloifa et al., 2020)). Similarly, the pH and acidity values trends over time agreed in olive brines from Spain (Medina et al., 2013) and Greece (Chytiri, 2020). However, salt concentrations were higher than previously reported (Montaño et al., 2021; Posada-Izquierdo et al., 2021).

3.2. Validation of the L. plantarum qPCR assay

The specificity of the new 16S rRNA primer set (planF/planR) for *L. plantarum* was assessed *in silico* from a total of 32 different *recA* sequences (*L. plantarum* strains, phylogenetically related genera, and bacteria commonly found in dairy products) retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/). No amplification capacity over non-targeted sequences was predicted (two mismatches per primer were allowed, Fig. S1a). Furthermore, *in vitro* specific amplification was determined by PCR (Fig. S1b). A TOPO TA plasmid (Invitrogen, USA) carrying the corresponding planF/planR *L. plantarum recA* insert (99.7% identity to partial *recA* gene of *L. plantarum*) was selected as a standard. Standard curves of log copy number (2×10^8 to 2×10^2 gene copies/μL) vs the C_t values (4–28) had a mean R^2 value of 0.99 (efficiency: 80.47–92.05%). Finally, a single melting peak was observed for the standards and all brine samples. Therefore, the qPCR approach designed in this study resulted in an effective and specific amplification of *L. plantarum*, making the new primers a useful tool to assess their abundances in olive brines.

3.3. Evolution of total bacteria and L. plantarum populations through fermentation

The absolute abundances of total *Bacteria* and *L. plantarum* are displayed in Fig. 1. Independently of the fermenting vessel, the highest values of total bacterial and *L. plantarum* populations were detected after 30 days of fermentation (average values 6.14×10^{13}, 4.21×10^{13}, and 1.95×10^{13} 16S rRNA gene copies/L of brine and 9.18×10^8, 1.38×10^{10}, 7.23×10^8 *recA* gene *L. plantarum* copies/L of brine for vessels A, B, and C, respectively). After day 30, the total bacterial and *L. plantarum* populations decrease steadily and significantly during all the fermentation period (up to 3.26×10^{12}, 1.34×10^{13}, and 7.46×10^{12} 16S rRNA gene copies/L of brine and 8.95×10^6, 7.24×10^8, and 2.07×10^8 *recA* gene *L. plantarum* copies/L of brine for vessels A, B, and C, respectively, at day 180). Besides, statistical differences were found for the gene copy numbers of both populations among the different vessels for a given time.

To the best of the author's knowledge, this is the first study using a qPCR approach to determine the absolute abundance of total bacterial populations in Spanish-style green table olive brines. This makes compare these results with those of previous works difficult due to the differences in the number of copies of the 16S rRNA in the genome of the different bacteria (Klappenbach et al., 2001). Nevertheless, Lucena-Padrós and Ruiz-Barba (2016) have previously described a sharp fall in the abundance of the bacterial communities at the end of the fermentation time by culture-dependent methods, which disagreed with the here reported as the bacterial abundance reduction was lesser than the described there.

On the other hand, there is only one copy of the *recA* gene in the genome of the different *L. plantarum* strains, according to the KEGG database, making possible a direct conversion of the *recA* genes copies to *L. plantarum* cells. Subsequently, the abundance of *L. plantarum* was similar to the values previously reported in Spanish-style olive brines by culture-dependent methods (Lucena-Padrós & Ruiz-Barba, 2019).

Therefore, the fermentation time had meaningful effects on the total bacterial abundance and the *L. plantarum* populations. Similarly, the origin of the vessels shaped the total bacterial and *L. plantarum* populations, indicating intra-specific differences among the three vessels. It is also important to note that the inoculum employed here has a high technological capacity as a broad quantity of *L. plantarum* was detected during the whole fermentation period.

3.4. Dynamics of bacterial communities over the fermentation time

The high-throughput sequencing of 16S rRNA generated a total of 4,071,850 high-quality bacterial sequences (average: 75,404 ± 4073 sequences per amplicon library) and 1776 different OTUs (average: 367 OTUs per amplicon library). The relative abundances (RA) of each OTU are shown in Table S1.

There were no statistical differences for the bacterial richness for the three vessels for a given sampling time, neither for a given time for the three vessels (Table S2). Therefore, the brine's origin and the fermentation time did not modulate the bacterial richness. Result in agreement

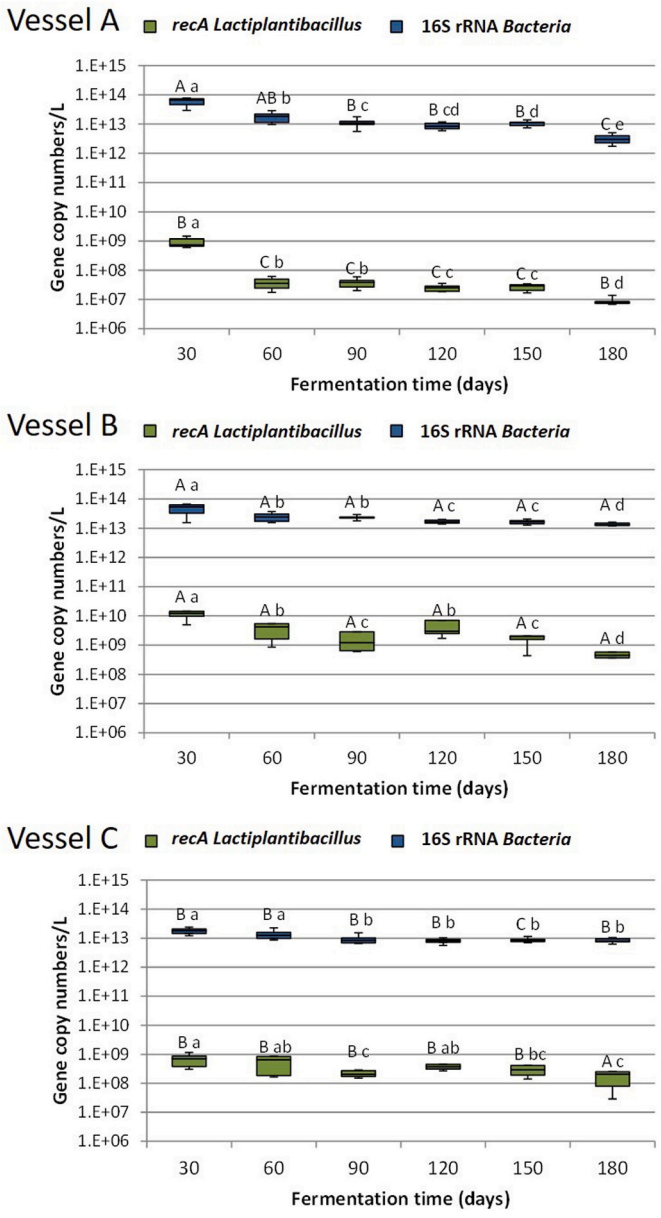

Fig. 1. Copy numbers/L of brine for the total bacterial and *L. plantarum* populations determined by quantitative PCR in olive brines samples (n = 3) retrieved from three independent vessels (A, B, and C). For a given population and vessel, lowercase letters indicate significant differences among sampling times, according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$), and capital letters indicate significant differences among vessels for a given sampling time.

with the previously described by De Angelis et al. (2015), which found a diversity richness in Grecian-style olive brines after 75 and 90 days of fermentation similar to the here reported. On the other hand, statistical differences were found for Simpson and Shannon diversity indices at different sampling times. Hence, the fermentation time affects the dominance and evenness of the bacterial communities of the brines.

The 1777 different OTUs were classified into phylum *Firmicutes* (mean RA 70.19%), *Proteobacteria* (mean RA 29.84%), the adventitious phylum *Actinobacteria* (RA < 0.02%), and the group of unclassified bacteria (mean RA 0.27%). *Firmicutes* was dominated by orders *Bacillales* (5.35%) and *Lactobacillales* (64.71%). *Proteobacteria* was constituted by eight different orders, with *Vibrionales* as dominant order (mean RA 29.79%) (Fig. 2, Table S3). The non-dominant orders were grouped in 'Minority Orders' (mean RA 0.01%).

There were several statistical differences in the RAs of the dominant phyla among vessels for a given time (RAs of *Bacillales* at 30, 60, 120, and 150 days of fermentation, RAs of *Lactobacillales* at 90, 120, and 150 days of fermentation, and *Vibrionales* at 30, 90, and 150 days of fermentation (Table S3)). Similarly, the RAs of *Bacillales* and *Lactobacillales* showed statistical differences among times for the three different fermenting vessels and *Vibrionales* only for containers A and B (Table S3). Therefore, both the fermentation time and the origin of the samples modulated the structure of the bacterial communities at the order level. No statistical differences were found for the RAs of Minority Orders among times or vessels.

The structure of the bacterial communities retrieved from olive brines at the order level were similar to those previously reported by culture-dependent methods (Lucena-Padrós et al., 2014; Rodríguez-Gómez et al., 2014) or by high-throughput sequencing techniques (De Angelis et al., 2015; Medina et al., 2018). Members of *Enterobacteriales* are often found in olive brines (De Angelis et al., 2015; Lucena-Padrós et al., 2014), although they can spoil the sensorial properties of fermented olives and are considered non-desired microorganisms in brines (Anagnostopoulos et al., 2020). Different works (Benítez-Cabello et al., 2020; De Angelis et al., 2015) have previously described the absence of *Enterobacteriales* when *L. plantarum* is used as a starter. Therefore, this study confirmed the inhibitory effect of *L. plantarum* over *Enterobacteriales* making this genus an excellent starter inoculum candidate to prevent and control the growth of spoiling microorganisms.

Fifty-six different groups were detected at the genus level. Thirteen genera had average RAs higher than 0.20%, and according to their mean RA (in decreasing order) were: *Vibrio* (29.79%), *Marinilactibacillus* (20.09%), *Lactiplantibacillus* (17.63%), *Enterococcus* (11.98%), *Secundilactobacillus* (5.56%), *Loigolactobacillus* (5.29%), *Amphibacillus* (4.66%), *Pediococcus* (1.55%), *Alkalibacterium* (1.09%), *Halolactibacillus* (0.79%), *Weissella* (0.78%), *Lentilactobacillus* (0.39%), and *Paucilactobacillus* (0.21%), plus the group of minority genera (0.18%) (Fig. 3). In general, the structure at the genus level were in agreement with those previous reports in other olives brines independently of the fermenting procedure (Benítez-Cabello et al., 2020; De Angelis et al., 2015; Kazou et al., 2020; Lucena-Padrós et al., 2014; Medina et al., 2018; Rodríguez-Gómez et al., 2014).

There were several statistical differences for the RAs of these dominant phyla among sampling vessels and times (Table S4). *Alkalibacterium* and the minority genera group were the only whose RAs were similar among the three containers for all the sampling times. Similarly, RAs of *Alkalibacterium* for vessel B, *Amphibacillus* for B and C, *Enterococcus* for vessel C, *Halolactibacillus* for vessels A and B, *Lactiplantibacillus* for vessel C, *Loigolactobacillus* for vessel C, *Marinilactibacillus* for vessel B, *Vibrio* for vessel C, *Weissella* for A and B, and the minority genera for the three vessels were not different among sampling times for a given container. Therefore, the sampling time and the vessel origin shape the structure of the bacterial communities in the olive brines without drastic changes in bacterial diversity. Result in agreement with Lucena-Padrós and Ruiz-Barba (2019), which found that the bacterial communities in brines from different table olive retrieved from South-Spanish factories were very similar after three weeks of fermentation. However, the same authors (Lucena-Padrós & Ruiz-Barba, 2016) found drastic differences in the structure of the bacterial communities at the end of the fermentation. Further research is necessary to confirm the likeness of the structure and the total abundance of the bacterial communities for the fermentation period, as reported in this study.

The common bacterial core was formed by all the dominant genus, representing 96.10% of the total sequences, except *Halolactibacillus*, *Lentilactobacillus Paucilactobacillus*, *Pediococcus*, and *Weissella*. Also, it is outstanding that the bacterial core was present in the olive brines during the whole fermentation period, despite using a starter culture. Subsequently, the bacterial community members were selected by the high stringent conditions imposed at the initial fermentation stages in some

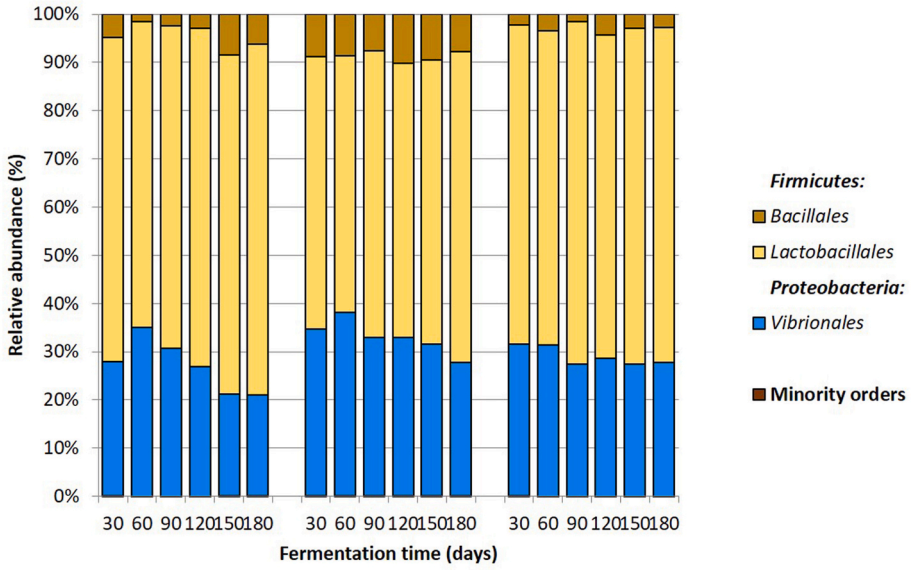

Fig. 2. Relative abundance (%) of the main bacterial orders (contribution >0.02%) obtained by Illumina sequencing analysis through the fermentation time in Spanish-style green table olive brines in the independent fermentation vessels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

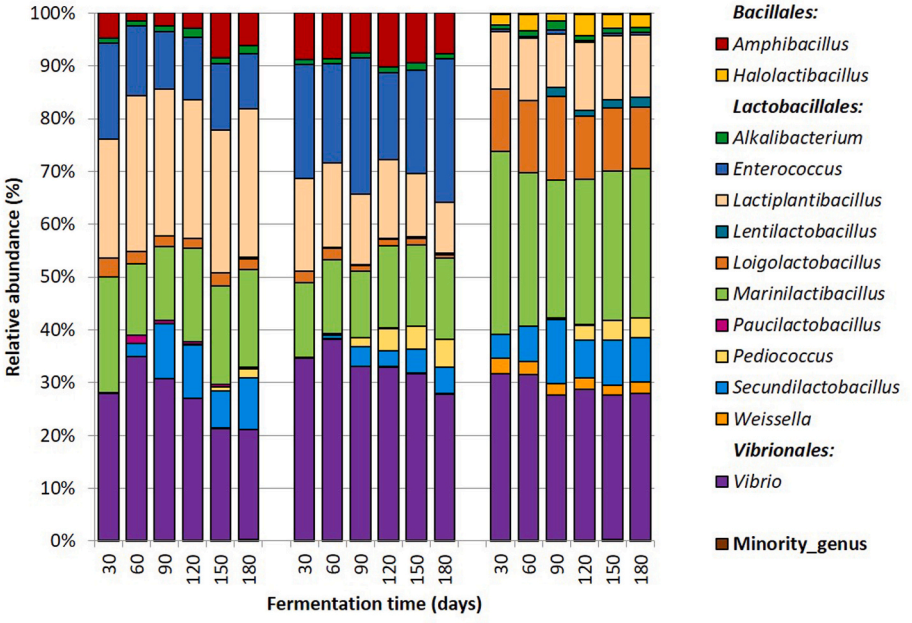

Fig. 3. Relative abundance (%) of the dominant bacterial genera (contribution >0.02%) obtained by Illumina sequencing analysis through the fermentation time in Spanish-style green table olive brines in the independent fermentation vessels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

way and maintained for all the fermentation period.

3.5. Diversity of lactobacilli in the olive brines

To deeper analyse the diversity of the different OTUs classified within the family *Lactobacillaceae*, a phylogenetic analysis of these OTUs with RAs >0.01% was made. According to the phylogenetic tree shown in Fig. 4 (sequences used for its construction are listed in Table S5), several phylogenetically distant lactobacilli were found in the olive brines used in this work. Therefore, the use of *L. plantarum* as a starter did not affect the colonisation and survival capacity of a plethora of members within the family *Lactobacillaceae*. It has been proposed that fermented olives are functional food involved in promoting human health due to the high level of probiotics (Abouloifa et al., 2020; Mu et al., 2018). In this regard, this work highlighted that started Spanish-style green table olives could constitute a significant source of beneficial LAB. Besides, the use of *L. plantarum* as a starter is a beneficial controller of non-desired microorganisms allowing the proliferation of other beneficial microorganisms.

The *L. plantarum* strain used in this work (OTU00002) was linked to the *L. plantarum* group (*L. plantarum*, *Lactiplantibacillus paraplantarum,* and *Lactiplantibacillus pentosus*). However, some discrepancies were found between the OTUs belonging to the remaining dominant lactobacilli (*Lentilactobacillus*, *Loigolactobacillus*, *Paucilactobacillus*,

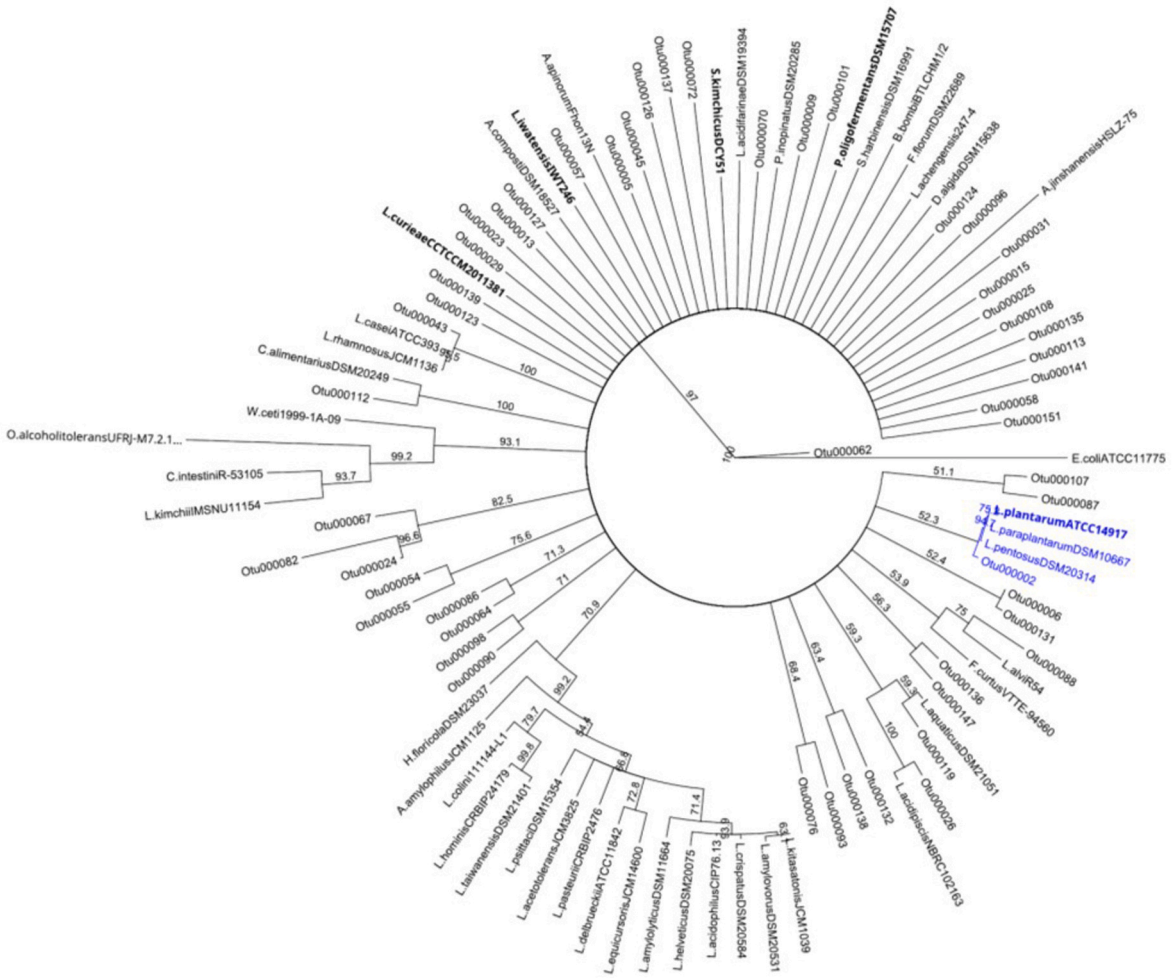

Fig. 4. Tamura-Nei phylogenetic tree of the OTUs within the family *Lactobacillaceae* retrieved from Spanish-style green table olive brines. The node of the starter culture (OTU00002, *Lactiplantibacillus plantarum*) was colored in blue, and the type strains of the dominant genera within *Lactobacillaceae* are black boldfaced. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

Secundilactobacillus) and their corresponding type strains. Other authors (Hill et al., 2018; Sun et al., 2015) previously highlighted some taxonomically discrepancies of lactobacilli under an exclusive 16S rRNA gene analysis due to high sequence similarities across the different lactobacilli. Additionally, the 16S rRNA Illumina sequencing resulted in a higher proportion of *Lactiplantibacillus* than those obtained by the *recA* qPCR approach, suggesting that the 16S Illumina approach was less accurate for classifying different lactobacilli at the species level. Finally, the *recA* gene's divergence among the different bacteria within the *Lactiplantibacillus* genus is wider than the observed for the 16S rRNA gene (86.4% vs 99.8% pairwise identity, respectively, data not shown). This could allow that other strains related to *Lactiplantibacillus*, such as *L. paraplantarum* and *L. pentosus* clustering next to the OTU00002 when a 16S rRNA approach was used, overestimating the abundance of this genus. Therefore, further research based on the whole genome shotgun sequencing is necessary to fill these disappointments in the phylogeny of lactobacilli.

To the best of the author's knowledge, this is the first work that analysed the bacterial diversity in olive brines under the recent reclassification of the family *Lactobacillaceae* proposed by Zheng et al. (2020).

3.6. Metabolic and functional roles of the dominant genera in olive brines

The dominant genera's metabolic and functional capacities are proposed according to their lifestyles described in previous scientific studies.

The genus *Vibrio* (phylum *Proteobacteria*, order *Vibrionales*) is composed of halophilic and alkaliphilic facultative anaerobes commonly isolated from aquatic environments (Farmer et al., 2015), often found in olive brines (Lucena-Padrós & Ruiz-Barba, 2019; Medina et al., 2018; Posada-Izquierdo et al., 2021). The high pH and salt of the brine form an ideal environment for *Vibrio*'s growth (Häse & Barquera, 2001). Besides, *V. olivae* was firstly isolated in this habitat (Lucena-Padrós et al., 2015). The fermenting capacity of *Vibrio* occurred via mixed organic acid production (Shieh et al., 2000), often related to a decrease in the sensory quality of the final fermented product. Hence, a high abundance of *Vibrio* is undesired in olive brines. The over-dominance of *Vibrio* in olive brines with a high level of salt (ca. 8%, similar to that observed in this work) compared with other less salty (around 5–6%) was previously reported (Lucena-Padrós et al., 2016). This lends support to that, the dominance of *Vibrio* in these olive brines could be linked to the high salinity to which the olives were subjected.

Marinilactibacillus (phylum *Firmicutes*, order *Lactobacillales*) is a LAB able to grow under hypersaline conditions (up to 15% NaCl) and pH values from 6.0 to 10.0 and initially isolated from marine organisms (Ishikawa et al., 2003). Its presence in olive brines have reported in other works (Benítez-Cabello et al., 2020; Lucena-Padrós & Ruiz-Barba, 2016). Furthermore, this bacterium can grow by utilising lactate when carbon sources are limited in a synergistic metabolism with other LAB (Suzuki et al., 2021).

The most abundant bacteria within the family *Lactobacillaceae* was *Lactiplantibacillus* (phylum *Firmicutes*, order *Lactobacillales*). It is a non-spore-forming facultatively anaerobic fermenter able to realise a lactic acid fermentation under a high content of available carbohydrates (Kleerebezem et al., 2003). The partial 16S rRNA Illumina sequencing shown that genus *Lactiplantibacillus* dominated over other LAB as expected due to the use of this genus as a starter. Several authors have previously found this bacterium in brines from both Spanish-style olives (Blana et al., 2014; Lucena-Padrós et al., 2019) and in other types of fermented olives (De Angelis et al., 2015; Kaltsa et al., 2015). In addition, several former members of the genus *Lactobacillus* were found in this work: *Halolactibacillus*, *Lentilactobacillus*, *Loigolactobacillus*, *Paucilactobacillus*, and *Secundilactobacillus*. Generally considered, lactobacilli can reduce the pH of the brines via lactic acid fermentation, preventing contamination of non-desirable microbiota and improving the organoleptic characteristics of the product and stabilising the final product (De Angelis et al., 2015; Sabatini et al., 2008).

Enterococcus (phylum *Firmicutes*, order *Lactobacillales*) is widely distributed in nature, and most of the strains within the genus are salt-tolerant (as high as 6.5%) (Díaz-Perez et al., 2010). Although this genus is a potential pathogen, it is also a well-known LAB. Its presence is widespread in fermented products from Mediterranean countries, such as cheese (Vandera et al., 2019) and fermented olives (Rehaiem et al., 2016). This genus produces enterocins with antagonistic activity against pathogenic bacteria (Braïek et al., 2017). Also, this genus promotes beneficial sensory characteristics and improves digestibility (Graham et al., 2020).

Amphibacillus (phylum *Firmicutes*, order *Bacillales*) is a slightly halophilic bacterium (unable to survive under 6% of NaCl), firstly isolated from composts of manure with grass and rice straw (Niimura et al., 1990). The presence of this genus in olive brines is not well-established (only described in olive brines by Lucena-Padrós and Ruiz-Barba (2016).

Pediococcus (phylum *Firmicutes*, order *Lactobacillales*) has been described as LAB genera previously related to olive brines (Abriouel et al., 2012) and other fermented foods (Yang et al., 2019). This genus can produce pediocins with a broad spectrum of antimicrobial activity against pathogenic Gram-positive bacteria making this genus of technological interest in fermented foods (Porto et al., 2017).

Alkalibacterium (phylum *Firmicutes*, order *Lactobacillales*) can survive at high alkalinity (pH from 9.0 to 12.0) and extreme salinity (Wang et al., 2016), and this genus has been related to olive fruit environments (Anagnostopoulos et al., 2020; Benítez-Cabello et al., 2020).

Halolactibacillus (phylum *Firmicutes*, order *Lactobacillales*) is a halophilic and alkaliphilic bacterium, firstly described in marine organisms (Ishikawa et al., 2005). Different authors have described the presence of this genus in olive brines (Lucena-Pradós et al., 2016; Portilha-Cunha et al., 2020). Also, this bacterium can produce exopolysaccharides with potential antioxidant activities against free radicals, and it can be exploited as a natural alternative for commercial antioxidants in olive brines (Arun et al., 2017).

Weissella (phylum *Firmicutes*, order *Lactobacillales*) is an obligately heterofermentative LAB, firstly isolated from fermented sausages (Collins et al., 1993). Several strains of *Weissella* have a high tolerance to NaCl (up to 14%) (Fusco et al., 2015), and its presence in olive brines has been previously reported (Lucena-Padrós et al., 2014; Medina et al., 2018).

3.7. Interaction of bacterial communities and their relationships with the physicochemical properties

There was a total of 84 pairs of meaningful correlations ($r > 0.3$), and 40 of them were positive, and 44 were negative (Fig. S2). It is worth noting that only one cluster was observed in the co-occurrence network among the 14 nodes, highlighting the imbricated bacterial interactive network developed in olive brines (Fig. 5). The most densely connected genera were *Halolactibacillus* (5 positives and 3 negatives strong correlations), *Marinilactibacillus* (4 positives and 4 negatives strong correlations), *Secundilactobacillus* (5 positives and 3 negatives) and *Weissella* (4 positives and 4 negatives). Subsequently, these groups were keystone of the olive brine bacterial community. On the other hand, the group of

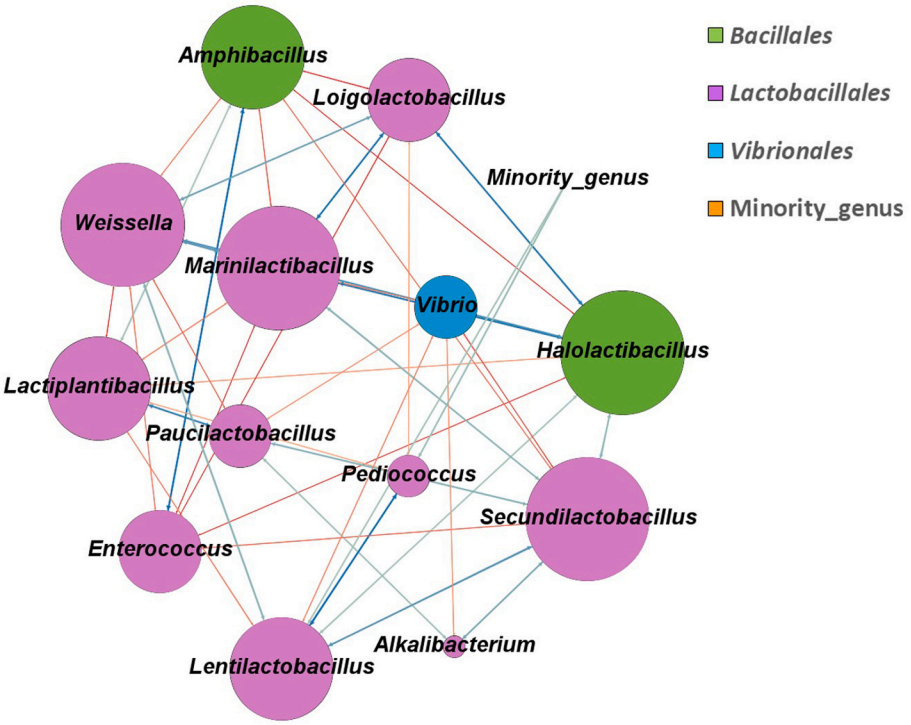

Fig. 5. Co-occurrence network of Spearman correlations of the structure of the dominant bacterial genera (mean: RA > 0.02%) in olive brines. The size of each node is proportional to the number of interactions; blue and red edges correspond to positive and negative Spearman's correlation coefficients, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

minority genus had the lowest levels of interaction (only 2 positives), suggesting that they are not essential members for the global bacterial community.

The NMS analysis of the dominant bacterial population in the olive brines and their physicochemical properties (pH, acidity, and NaCl concentration) demonstrated that bacterial communities were clearly separated into three clusters according to their vessel origin without clear relational patterns with sampling time (Fig. 6). Therefore, there were some intra-specific changes in the bacterial structure among the different vessels without apparent changes due to the sampling time, confirming that the specific pH, acidity, and NaCl concentration values from each container modulated the bacterial diversity. Hence, the brines' origin modulated the structure of the bacterial communities without drastic changes. Considering that the fermentation occurred independently in each of the three containers analysed, a high divergence in the diversity could be expected; however, the biodiversity was circumscribed to the same specific groups in the different vessels. In this regard, Lucena-Padrós and Ruiz-Barba (2016) have previously stated that the strength conditions employed in the fermentation drove bacterial diversity among brines taken from different containers in the same 'patio'.

On the other hand, it is outstanding that the NaCl concentration was the main parameter that influenced the structure of the bacterial communities (Table S6). Result that agreed with several works that have previously described that the salinity is a priming force for the development of bacterial communities in different fermented foods, as are kimchi (Lee et al., 2018), and the Chinese traditional fermented vegetables 'paocai' (Zhao et al., 2020).

Finally, the Spearman correlations analysis revealed a large number of pairwise correlations among physicochemical properties and the abundance of total bacteria and *L. plantarum*, biodiversity indices, and RAs of the dominant genera (Fig. 7 and Table S7). In this sense, there were different strong positive correlations between NaCl concentration and Shannon index and RAs of *Halolactibacillus*, *Lentilactobacillus*, *Marinilactibacillus*, *Pediococcus*, *Secundilactobacillus*, and *Weissella*. However, NaCl was negatively correlated with the gene copies of bacterial 16S rRNA, *L. plantarum recA*, Simpson index values, and RAs of *Amphibacillus*, *Enterococcus*, *Lactiplantibacillus*, and *Vibrio*. Hence, these results lend support to that the NaCl concentration was a pivotal driving force of the bacterial community, modulating both bacterial abundance and structure, as described above for the NMS analysis (Fig. 6). These results agreed with Tassou et al. (2002), which clarified that the differences in NaCl levels of brines produce several changes in black olives' microbiological and physicochemical characteristics.

The present work provided novel insights into bacterial communities in olive brines inoculated with *L. plantarum* in three independent vessels over six months of fermentation. In general, these results highlighted that the use of *L. plantarum* as a starter has no detrimental effects on the structure of the bacterial communities retrieved from Spanish-style green table olive brines. Besides, the use of *L. plantarum* allows the colonisation of brines by several LAB, including members of other members of the *Lactobacillaceae* family. Furthermore, several relationships among the physicochemical properties and the microbial community were found.

4. Conclusions

The proposed *L. plantarum recA* qPCR assay is a useful molecular tool to quantify this species in olive brines.

The application of *L. plantarum* in the brines promoted an optimal development of bacterial communities as determined by bacterial abundances and biodiversity.

Despite the use of the starter, several LAB strains (including the different new genera within *Lactobacillaceae*) were able to colonise the olive brines.

L. plantarum has a meaningful technological capacity in the fermentation of olives, and it is a good stater that can be used to control spoiling microorganisms.

The specific conditions in each fermented container shaped the bacterial community structure and influenced the total bacterial abundances and *L. plantarum* populations.

Finally, the NaCl concentration was a driving force of the bacterial communities, modulating the size of total bacterial and *L. plantarum* populations and shaping the structure of the dominant bacterial genera.

CRediT authorship contribution statement

D. Correa-Galeote: Formal analysis, Investigation, Term, Conceptualization, Formal analysis, Writing – review & editing, Visualization, Supervision. **I. Ghomari:** Investigation, Methodology, Writing – original draft, Visualization. **A. Asehraou:** Term, Conceptualization, Writing – review & editing, Visualization. **J. González-López:** Funding acquisition, Formal analysis, Supervision, Term, Conceptualization, Resources, Writing – review & editing, Visualization.

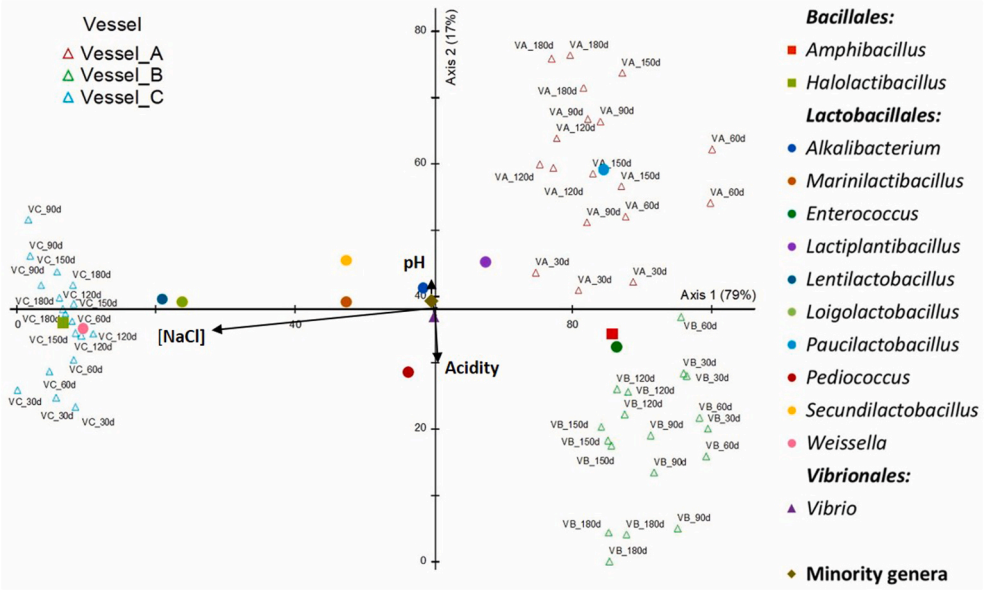

Fig. 6. Nonmetric multidimensional scaling (NMS) ordination of the structure of the bacterial dominant genera (RA > 0.02%) in olive brines (n = 3) retrieved from the three independent fermentation vessels during the fermentation period and their linking to the abiotic variables influencing the brines (pH of brines, pH; titratable acidity in brines, acidity; NaCl concentration in brines, [NaCl]). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

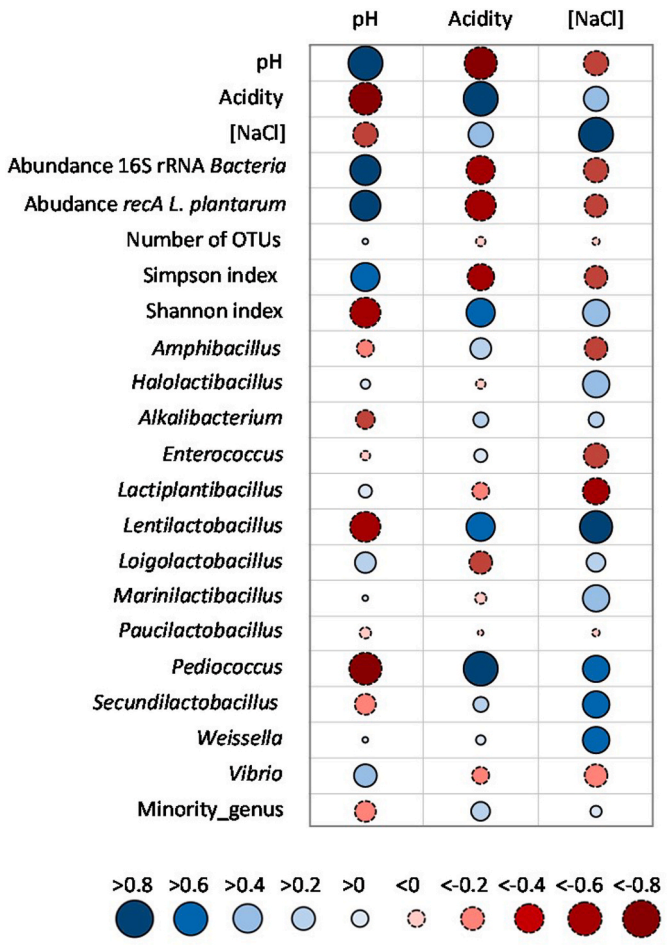

Fig. 7. Spearman's correlations among pH, titratable acidity, and NaCl concentration and the values of the remaining variables in the olive brines used in this work (total *Bacteria* and *L. plantarum* abundances, number of OTUs, Simpson, and Shannon index values, and RAs of the dominant genera (RA > 0.02%)). Positive and negative correlations are represented by solid and dashed lines, respectively. The size and colors of the circles represent the robustness of the correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

Declaration of interests

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113212.

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