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The use of multifunctional yeast-lactobacilli starter cultures improves fermentation performance of Spanish-style green table olives

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Running title: Multifunctional table olive starters

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Abstract

In this work, *Lactobacillus pentosus* LPG1, *Lactobacillus pentosus* Lp13, *Lactobacillus plantarum* Lpl15, and *Wickerhanomyces anomalous* Y12, all of them previously isolated from fermented table olive biofilms, were used (alone or in combination) as multifunctional starters for Manzanilla Spanish-style green table olive fermentations. Their performances were evaluated through the changes in the key physico-chemical and microbiological parameters, correlation between AI-2 production and biofilm formation, inoculum imposition, metataxonomic analysis and sensory characteristics of the finished products. Inoculation only with lactic acid bacteria (LAB) strains led to higher titratable acidities and lower pH values than the spontaneous fermentation (non-inoculated control), mainly during the first steps of processing. However, the sequential inoculation of the yeast and then the combination of the 3 LAB strains showed the most favourable evolution. LPG1 strain and, particularly Lp13, were excellent biofilms former and showed the major imposition on the fruit epidermis, as corroborated by rep-PCR analysis. Production of AI-2 was lower in the treatment inoculated exclusively with yeast Y12 but had the highest presence in the sequential yeast-LAB inoculum, with its maximum concentration and maximum LAB population on fruits (19 th days) strongly related. Metataxonomic analysis of the biofilms at the end of the fermentation revealed, in addition to *Lactobacillus*, high proportions of sequences from genera *Marinilactobacillus, Alkalibacterium*, *Halolactobacillus,* and low levels of *Halomonas* and *Aerococcus*. Compositional data analysis of the omics data revealed that Lpl15 was scarcely efficient for controlling the spontaneous microbiota since its treatment presented the highest proportions of *Aerococcus* genus. Finally, the sensory analysis showed similar characteristics for the treatment inoculated with LPG1 and the spontaneous process, with olives inoculated with the yeast (alone or in combination with *Lactobacillus* strains) showing attractive scores. Then, inoculation of Spanish-style table olive fermentations with a sequential yeast and LAB combination could be an advisable practice.

Keywords: Autoinducer; Biofilm; Compositional analysis; Multifunctional starters; metagenomic analysis; Sensory analysis; Table olives.

1. INTRODUCTION

Table olive is widespread around the world and represents an important economic source for many countries, with an estimated production that currently exceeds 2.7 million tonnes/season (IOC, 2019). Their products are among the most important and well known fermented vegetables of the food industry, especially in the Mediterranean basin. The most common commercial processing are: a) alkali-treated green olives (Spanish style), b) black ripe olives by alkaline oxidation (Californian style), and c) directly brined olives (Greek style) (Garrido-Fernández et al., 1997).

In Spanish-style green table olives, the microorganisms responsible for the fermentation are the lactic acid bacteria (LAB), who produce lactic acid and reduce the pH, albeit yeasts can also provide interesting technological features (Arroyo-López et al., 2012a). The most frequently LAB species found are *Lactobacillus pentosus* and *Lactobacillus plantarum*, together with yeasts of the genera *Pichia, Saccharomyces, Candida, Wickerhanomyces,* and *Debaryomyces* (Arroyo-López et al., 2008; 2012a; Ruiz-Barba et al., 1994; Bautista-Gallego et al., 2010; Randazzo et al., 2011; Hurtado et al., 2012; Cocolin et al., 2013; Tofalo et al., 2014).

The spontaneous fermentation is driven by the autochthonous microbiota from the environment or the raw material; however, spoiling microorganisms such as *Enterobacteriaceae, Clostridium* and *Propionibacteriaceae* may appear, producing quality deterioration, food safety risks, and substantial economic losses. To prevent their growth, the application of starter cultures, mainly belonging to *Lactobacillus* genera (Arroyo-López et al., 2012a; Hurtado et al., 2012; Randazzo et al., 2014) and yeast species (Arroyo-López et al., 2012a; Bevilacqua et al., 2013; De Angelis et al., 2015; Tufariello et al., 2019) could be an advisable practice.

Currently, there is a tendency for the selection of microorganisms not only with good technological properties but also with probiotics characteristics. Among others, Blana et al. (2014) and Rodríguez-Gómez et al. (2013, 2014) used multifunctional *L. pentosus,* and *L. plantarum* strains, isolated from industrial brines, as a starter cultures. Both groups reported the ability of these organisms to colonize the olive surface and form biofilms. Recently, Benítez-Cabello et al. (2019a) studied *in vitro* the multifunctional properties of LAB strains isolated directly from processed fruits. Bonatsou et al. (2018) and Porru et al. (2018) evaluated the multifunctional properties of yeasts from Kalamata and Bosana natural black olive fermentations, respectively, suggesting the use of yeasts, individually or mixed with LAB, as starter cultures for table olive processing. Particularly, the simultaneous inoculation may increase the population of bacteria since the yeasts produce vitamins, amino acids, or can split complex carbohydrates into simpler sugars, which are essential for the growth of *Lactobacillus* spp. (Arroyo-López et al., 2008, 2012a). Also, the use of sequential yeast-*Lactobacillus* starters increases the volatile composition of Spanish-style green table olive fermentations (Benítez-Cabello et al., 2019b).

This work aims validation of the technological properties of potential probiotics lactobacilli strains, isolated from table olive biofilms, and a yeast, independently or in sequential inoculation, through assessing their: i) fermentation performance, ii) imposition, iii) production of auto-inducer 2 (AI-2) and biofilm formation, iv) influence of the inoculum on the total bacterial biodiversity present in the fruit biofilm, and v) influence on the sensory characteristics.

2. MATERIALS AND METHODS

2.1. Olive processing

Fruits (Manzanilla *cv*) were processed according to Spanish-style during the 2017/2018 season using cylindrical fermentation vessels (5 L liquid + 9.5 kg olives). Fruits were debittered for 7 h with a 3.2% NaOH solution at 20ºC, containing 2.2 % NaCl and 0.89% $CaCl_2$ (97% purity). Then, olives were immersed in tap water for 5 h, to reduce the excess of alkali, and, after removing it, brined in a solution containing 12.0% (w/v) NaCl, 0.13% $CaCl_2$, and 0.08% HCl (to correct the initial pH). Fruits were processed at the industry and immediately transported to the pilot plant of Instituto de la Grasa (CSIC, Seville, Spain) where the inoculation experiments were performed.

2.2 Inoculum treatments

L. pentosus LPG1 (onwards LPG1), *L. pentosus* Lp13 (Lp13), *L. plantarum* Lpl15 (Lpl15), and the yeast *Wickerhanomyces anomalus* Y12 (Y12) were used as starter cultures. All of them were isolated from the surface of table olives and belong to the Table Olive Microbial Collection of Instituto de la Grasa (TOMC). LAB strain selection was based on diverse *in vitro* phenotypic tests related to their probiotic and technological potential (Benítez-Cabello et al., 2019a). Yeast Y12 was selected based in their high lipase, esterase and β-glucosidase activities, high survival to gastric and pancreatic digestions and cholesterol removal (Rodríguez-Gómez et al., 2012a).

The experimental design consisted of individual inoculation of each organism (T1, for LPG1; T2, for Lp13; T3, for Lpl15; T4, for Y12), a sequential inoculation of them (T5, Y12 followed by LPG1+Lp13+Lpl15), and a non-inoculated (spontaneous) fermentation (T6). All experiments were executed in duplicate at a temperature which ranged, approximately, from 29ºC (September 2017) to 16ºC (December 2017). The processes were monitored for 65 days.

Bacteria and yeast inocula were grown overnight on Man, Rogosa and Sharpe (MRS) broth medium (Oxoid, Basingstoke, Hampshire, England) at 37ºC and YM broth (Difco, Le Pont de Claix, France) during 48 h at 28ºC, respectively. Cultures were then washed and re-suspended in 0.9% sterile saline buffer. The inoculation was achieved at the 9 th day after brining to reach an initial population level of approximately 7 log $_{10}$ CFU/mL in the cover brine for LAB strains. In case of the sequential starter culture (T5), the yeast inoculation was performed at the 1 st d after brining, and the combination of bacteria 8 days later, with the initial yeast populations expected as 5 $\log_{10}$ CFU/mL and 7 $\log_{10}$ CFU/mL (the combination of LAB). At the moment of the inoculation, the pH and NaCl of brines were about 6 units and 7% (w/v), respectively.

2.3. Physicochemical analyses

Determinations of titratable, combined acidity, pH, and salt in the fermentation brine were carried out using the methodology described by Garrido-Fernández et al. (1997). Reducing sugars (sucrose, glucose, fructose, and mannitol), organic acids (lactic, acetic, and citric), and ethanol in the cover brines were determined by HPLC according to the protocols described by Rodríguez-Gómez et al. (2012b).

2.4. Microbiological analyses

Microbial populations adhered to the surface of the olives were recovered at the 1 st, 19th, and 65th day of fermentation according to the methodology described by Benítez-Cabello et al. (2015). The operation consisted of the disintegration of biofilms with stomacher and collecting microorganisms in 0.9% sterile saline buffer. Brine samples were withdrawn at the 0, 1, 2, 5, 9, 13, 19, 35 and 65 days. Appropriate dilutions of the saline buffer (for biofilms) or brine were plated on the appropriate medium, using a Spiral Plating System model dwScientific (Don

Whitley Sci. Ltd., Shipley, U.K). *Enterobacteriaceae* were plated on VRBD (Crystal-violet Neutral-Red bile glucose) agar (Merck, Darmstadt, Germany), LAB on MRS agar supplemented with 0.02 % sodium azide (Sigma, St. Luis, USA), and yeasts on YM (yeast-mal-peptone-glucose) agar (Difco™, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate as selective agents. Plates were incubated at 37ºC for 24h and 48h for *Enterobacteriaceae* and LAB respectively or 30ºC during 48 h for yeasts.

2.5 Biofilm observation

Scanning electron microscope (SEM) techniques were used to corroborate the presence of biofilms in the fruits at 35 days of fermentation. For this purpose, fruits were previously treated according to the protocol of Kubota et al. (2008). Firstly, the non-adhering cells were removed from the olives by washing them twice for 1 h with a 100 mM phosphate buffer (pH 7.0). Olives were then placed for 2 h in a solution composed of the same phosphate buffer with 5% glutaraldehyde. Afterwards, fruits were dehydrated in increasing concentrations of ethanol (from 50 to 100%). Then, samples were placed in 2-methyl 2-propanol for 20 min, and slices (0.5 cm^2) of the fruit epidermis were fixed onto glass slides. Lastly, olive slices were sputtered with gold using a Scancoat Six SEM sputter coater (Edwards, Gat, Israel) for 180 s. Finally, they were observed with an SEM model JSM-6460LV (Jeol Ltd, Tokyo, Japan).

2.6. Autoinducer-2 bioassay

Auto-inducer-2 (AI-2) activity in fermented olive biofilm was determined at the 1 st, 5th, 9th, 19th, and 65th day of fermentation. For this purpose, biofilms were detached according to the protocol described by Benítez-Cabello et al. (2015). Next, they were centrifuged at 10,000 *g* at

4 ºC for 10 min and the supernatants were filter sterilized through a 0.22 m-pore-size filter (Millipore Ibérica, Madrid, Spain). Then, AI-2 activity was determined by measuring the bioluminescence response of *Vibrio harveyi* as previously described by Vilchez et al. (2007). Analysis traces of AI-2 requires a standardization of the *V. harveyi* bioassay (Vilchez et al., 2007). Briefly, a stock culture of *V. harveyi* MM32 (Km ^{r}, *luxLM*::Tn5, *luxS*::Tn5; AI-1 $^{-}$; AI-2^{-}) (courtesy of Bonnie Bassler) was streaked on auto-inducer bioassay (AB) plates, incubated overnight at 30 ºC, and then bacteria from agar plates were resuspended in AB broth to give a pre-inoculum, which was then diluted several times in AB broth to give a working solution of the sensor strain MM32. Then, 180 L of working solution plus 20 L of the supernatants from biofilms were placed into wells of black microtiter plates (Nunc Microwell, Thermo Fisher Scientific, Roskilde, Denmark) and bioluminescence measurements were taken every 30 min for 24 h with Victor X3 (2030 Multilabel Reader, Perkin-Elmer). Eight replicates were measured for every sample. Results were expressed as counts per second (CPS).

2.7. Genotyping of LAB population

A total of 10 LAB colonies from the diverse treatments were randomly obtained at the end of the fermentation process (65 days), grown in MRS broth at 37ºC for 48 h and stored at -80ºC in 20% glycerol (v/v) until further analysis. Prior genotyping, DNA of the 60 LAB isolates was extracted from 1 mL of early culture (OD_{600nm}= 1.0) with the rapid chloroform: isoamyl alcohol method described by Ruiz-Barba et al. (2005), and further amplified by rep-PCR analysis using the GTG_5 primer and protocol described by Gevers et al. (2001). The resulting fingerprints were digitally captured and analysed with the Bio-Numerics 6.6 software package (Applied Maths, Kortrijk, Belgium). Only bands representing amplicons between 100 and 3,000 bp in size

were included in the analysis.The dendrogram was generated by the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm, setting a value of 0.5% optimisation and 1.25% curve smoothing. The similarity among digitalised profiles was calculated using the Pearson product-moment correlation coefficient. A similarity coefficient of 87.5% was considered as a cut-off value to discriminate between clusters. This cut-off value was chosen by using LPG1, Lp13, and Lpl15, which were included in all PCR reactions as an internal control for comparison.

2.8. Metataxonomic analysis

Total microbial genomic DNA was isolated and purified from biofilms at the end of fermentation (65 days) using the PowerFood® Microbial DNA Isolation Kit (MoBio, Carlsbad, Calif) according to the manufacturer instructions, and was stored at −20 °C until use. Biofilms DNA samples from the different treatments were sent for sequencing to FISABIO (Valencia, Spain). The gene-specific sequences used in this protocol target the bacterial 16S rDNA gene, both V3 and V4 regions (Klindworth et al., 2013). Libraries were sequenced using a 2x300 bp paired-end run (MiSeq Reagent kit v3 (MS-102-3001) on a MiSeq Sequencer, according to manufacturer's instructions (Illumina). For the bioinformatic analysis, the *.fna format files were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version 1.9.1. http://qiime.sourceforge.net/) in a server running Ubuntu v16.04. Operational Taxonomic Units (OTUs) were defined at the level of ≥97% sequence homology, using SILVA108 as reference sequence database (McDonald et al., 2012). The OTU tables were collapsed at five taxonomic levels (Phylum, Class, Order, Family, and Genus).

The metagenomics data (OTUs) were also analysed as compositional data (CoDa, a constant sum of observations or row) (Pawlowsky-Glahn, Egozcue, Tolosana-Delgado, 2015) and subjected to their special exploratory tools, which included variation array and *clr* transformation (which preserves the distances) (Thió-Henestrosa, Daunis-i-Estadella, 2011). In CoDa, the relative variability of X (matrix of metagenomic information, with treatments as rows and microorganism genera as parts) is given by the matrix of log-ratio variances, originally defined by Aitchison (1986) as:

$$T = \begin{pmatrix} t_{11}\, t_{12} \cdots t_{1D} \\ t_{21}\, t_{22} \cdots t_{2D} \\ \cdots \cdots \cdots \cdots \\ t_{D1}\, t_{D2} \ldots t_{DD} \end{pmatrix} \quad \text{where } t_{ij} = \operatorname{var}\left(\ln \frac{x_i}{x_j}\right)$$

Besides, the central log-ratio transformation (*clr*) is defined as:

$$Z = \operatorname{clr}(X) = \left[\ln \frac{x_1}{g_m(x)}, \ldots, \ln \frac{x_D}{g_m(x)}\right]$$

where the transformation is made observation-wise, with the denominator being the geometric mean of parts estimated across observation. The variability of each clr part (*clr* variance) is estimated part-wise from the Z matrix and transposed for vertical presentation at the end of the variation array matrix. The sum of the *clr* variances accounts for the total variance. The Z matrix was also used for building the metataxanomic CoDa biplot (Aitchison, & Greenacre, 2002). Finally, *clr* transformed data were also subjected to standard multivariate analysis and bicluster (heat map), using the MultBiplot R package (Vicente Villardón, 2016).

2.9. Sensory analysis

After fermentation, the brines from each treatment were replaced by a new one with the appropriate concentrations of salt and lactic acid to reach the following levels at equilibrium: 5% NaCl and 0.5% titratable acidity. After obtaining the equilibrium, the fruits from the different treatments were sensory analyzed.

Fruits were first evaluated by 9 trained members from Instituto de la Grasa (CSIC), in a standardized testing room, following the "Method for sensory analysis of table olives" described by IOC (2011). Descriptors related to the perception of negative sensations (defects in flavour or aroma) or fruits defect (spots, damage or browning) were used for the fermented olive classification according to the IOC (2011) while gustatory attributes (salty, acid, bitter), kinesthetic sensations (hardness, crunchiness), browning, brown spots and overall acceptability were used for the Quantitative Descriptive Analysis of the products. Four olives were presented to each panellist in a normalized glass (IOC, 1987). For the evaluation of the attributes, the panellists received a standardized profile sheet with a scale on which they should indicate (mark) the intensity of each attribute, with the extremes left and right representing the absence and maximum intensity values in terms of perception, respectively. The intensity ranged from 1 to 11, with the scale being 10 cm long. The intensity was measured from the origin of the segment with a precision of 0.1 by using a rule.

For R_{index} estimation, a second blind tasting was done with a total of 22 non-trained panellists. In this case, they were asked to compare each treatment with the non-inoculated control (T6), based on gustatory attributes, evaluating, in this case, whether each treatment liked more, equal or less, (safer or doubtful in all cases) than the non-inoculated control samples. Both duplicated evaluations were carried in two different days (Clift et al., 2000).

The sensory data were analysed using the SensoMineR v.1.07 software (Husson, & Lê, 2007). The program was designed and programmed in R language (R Development Core Team, 2011) and collects classical methods usually applied when analysing sensory data as well as others directly conceived by the developers. SensoMineR provides not only a synthesis of the results of the analyses of variance (ANOVA) models but also numerous easy to interpret graphical outputs. For estimation of the confidence limits of the centres of the treatments, SensoMineR produces a virtual panel, extracting successive samples (n=1000) from the original data. The ellipses are then built to include 95% of them. XLSTAT (2017) and Multibiplot R (Vicente Villardón, 2016) packages were also applied for comparison of results or specific tests.

3. RESULTS

3.1. Evolution of physico-chemical parameters through fermentation

The changes in the main physico-chemical characteristics during fermentation followed the typical trend for this table olive processing type (Figure 1). It was characterised by a fast pH decrease and free acidity increase during the first days of fermentation due to the rapid lactic acid production, which reached their maxima at around the 35th day of fermentation. However, a different evolution of pH and titratable acidity production among treatments was observed initially; the highest free acidity and lowest pH values (particularly at the 9 th day) were obtained in T5 treatment, inoculated with the sequential starter culture (Y12 and LPG1+ Lp13+Lpl15), followed by T1, T2, T3, T6 (control), and finally T4. Overall, T4 (inoculated only with the yeast species) showed the highest pH and lowest free acidity values, indicating less intense lactic acid fermentation. This way, the final pH ranged from 4.06 (±0.11) in T1 treatment to 4.37 (±0.09) in T4 treatment, while final free acidity ranged from 0.57 (±0.05) in T4 treatment to 0.81 (±0.01) g

lactic acid/100mL in T6 treatment. The evolution of combined acidity and NaCl concentration was very similar in all cases, showing a slight increased through fermentation, from initial 0.04 (±0.01) to final 0.15 (±0.01) Eq/l for the first and from 7.13 (±0.05) to final 7.47 (±0.21) % for salt.

Different rates of reducing sugar utilization were detected among treatments (see Table S1 in supplementary material) with glucose being preferably consumed in all treatments, followed by mannitol, fructose and saccharose (in T1, T4, and T5), or fructose, mannitol and saccharose (in T2, T3, and T6 (control)). Only the treatment inoculated with the yeast Y12 (T4) was able to use saccharose completely while this sugar remained almost unaltered in the rest of fermentations. Likewise, treatment inoculated with Lpl15 (T3) did not use the mannitol in brine. Inoculation with LPG1 strain (T1) was the most effective treatment in the consumption of total sugars (81.98% reduction), followed by T5 (79.45%). However, the high consumption of sugar observed in the T1 was not reflected as lactic acid at the end of the fermentation (16.64 g/L). The most top production of lactic acid was observed in T2 (Lp13) (18.69 g/L), followed by T3 (18.60 g/L), and T6 (18.34 g/L), whereas T4 (inoculated with yeast) led to the lowest lactic acid production (11.51 g/L). As expected, inoculum with only yeast (T4) led to highest ethanol production (0.89 g/L) since T6 (spontaneous), and treatments inoculated with only bacteria had low and similar average production (0.20 g/L). As expected, T5 (inoculated with the combination of bacteria and the yeast), produced intermediate ethanol content (0.36 g/L) (Table S2).

3.2. Microbial counts through fermentation

Enterobacteriaceae were never detected in brine or fruit. The highest values of LAB in brine (around 7-8 $\log_0$ CFU/mL) were detected from the 9th to the 19th day of fermentation,

albeit T4 (Y12) and T6 (spontaneous) only reached this level at the end of the period (Figure 2). After the 19th day, LAB counts in all treatments were similar (Figure 2) with a slightly decreasing trend, which reached around 6 $\log_{10}$ CFU/mL at the 65th day of fermentation. In fruits, only T3 followed by T1 had LAB one day after brining but at the 19th day of fermentation, all treatments reached the maximum population (around 7 $\log_{10}$ CFU/g). These values decreased to 6 $\log_{10}$ CFU/g on the 65th day of the process (Figure 2).

Regarding yeasts in brine, the initial population in T4 and T5 (both inoculated with Y12) was ~5 $\log_{10}$ CFU/mL (Figure 2), reach the maximum at about the 10th day, and decreased to stabilize after the 20th day. In the T1, T2, T3, and T6 (spontaneous), yeasts were detected at the 9th day (~3 $\log_{10}$ CFU/mL), increased up to the 20th day, and stabilized. Yeast population on fruit at the 20th day was lower in T2 and T5 than on the others but, at the 65th day, T5 had the highest level (5 $\log_{10}$ CFU/g) and T3 the lowest (3.5 $\log_{10}$ CFU/g) with the rest of treatments showing values within this interval.

3.3. AI-2 detection and biofilm formation

AI-2 activity was assayed in biofilms after different periods of fermentation (Figure 3). The highest value was found at the 9th day of fermentation in T5 treatment, while at the 19th day of fermentation (coinciding with the maximum LAB population on fruits) the lowest was found in T4 (inoculated with Y12), with similar slightly higher values in the rest of treatments. However, at the end of the process, there was a gradation in AI-2 between treatments with T6>T2>T1>T4=T5>T3. Interestingly, the highest value at this moment was observed in T6 (spontaneous) and the lowest in T3 (Lp13).

Finally, biofilm formation on fruit epidermis was confirmed *in situ* by SEM at the 35 th day of fermentation (Figure 4), finding that the microorganisms were strongly attached to the fruit surface, proving the ability of the inocula of yeast and LAB to migrate from brines to fruits and the simultaneous production of an exopolysaccharide matrix on the olive epidermis.

3.4. Genotyping of the LAB isolates in biofilms

Inoculum imposition was determined by rep-PCR with GTG_5 primer and clustering analysis for the LAB isolates obtained from the olive biofilms at the 65 th day of fermentation. Fingerprinting of randomly LAB isolates from the different treatments were compared with the profiles of LPG1, Lp13, and Lpl15 (Figure 5). The dendrogram generated showed the presence of two major groups. Only in T1 treatment, inoculated with LPG1 strain, the 100% of the LAB isolates shared a 94.75% similarity with LPG1 profile, whereas 100% of isolates obtained from the T2, T3, T4, T5, and T6 treatments were similar to the Lp13 profile (87.75% similarity). Then, the results show a considerable colonizing capacity of the Lp13 strain, as well as the absence of Lpl15 strain at the end of fermentation.

3.5. Metataxonomic analysis

The massive sequencing analysis generated a total of 2,312,472 raw sequences for the 12 samples while the sequences with high quality re-covered after the quality control were 2,280,200 (98.60%) and the cleaned sequences that were included to be assigned into OTUs were 1,301,286 (57.07%). Finally, after removing chloroplast and mitochondria, a total of 1,006,447 sequences (43.52%) were used for metataxonomic analysis with a mean of 83,870 sequences per sample.

The analysis of the fruits samples at the end of the fermentation revealed the presence of DNA belonging to 4 main genera. Sequences of *Marinilactibacillus, Halolactobacillus, Lactobacillus,* and *Alkalibacterium* were always found in high proportions, but with differences among treatments (Figure 6). *Lactobacillus* genus showed the highest proportion (31.06% sequences) in T5 (mixed inoculum) and the lowest (12.71%) in T2. *Marinilactibacillus* was found mainly in treatment T1 (59.98%). *Halolactobacillus* was present primarily (29.77%) in T2 while *Alkalibacterium* showed the highest-similar proportions in T3 (17.71%) and T4 (18.37%). *Aerococcus, Halomonas* and *Bacillaceae* family were also detected in the different treatments, but with low contributions (<1.50%).

As the sum of sequence frequencies of microbiota by treatment is constant (=100), the data can be considered CoDa, which carries relative information, with the log-ratios and their variances (variation array) playing a relevant role (Table 1). The variation array includes the log-ratio variances on the upper diagonal and the mean log-ratios in the lower. The greatest variance corresponds to log-ratio of *Aerococcus* over any of the others and to its *clr* transformed data which means this genus shows the greatest differences among treatments and may have the major power for segregation among treatments. A further approximation of the origin of the variance is provided by the covariance biplot (Figure 7A), where T3 (Lpl15) shows a particular trend, characterized by a large proportion of *Aerococcus*; that is, this inoculum had low efficiency for controlling the initial spontaneous microbiota. The graph also indicates single replicates of T1, T6, T4 (notably singular at the top) or T2 had individual microbial behaviours. Furthermore, the greatest log-ratio variances (≈the distance between their respective arrow ends) are observed between clr-*Aerococcus* and any of the others (mainly with respect to clr (centred

log-ratio transformation) *Halomonas*, clr-*Halolactobacillus* or even clr-*Lactobacillus*) (Figure 7A).

The simultaneous relationships between genera and treatments may be observed in the bicluster, built using *clr* transformed data (Figure 7B) where the singular microbiological behaviour of T3 is also observed. This treatment is characterized by the high (red colour) contribution of *Aerococcus* and slightly above neutral (brown) of *Alkalibacterium*, but, on the contrary, very low presence (green) of the rest of the bacteria. To notice also the particular cluster of one T4 replicate abundant in *Lactobacillus* and *Alkalibacteria* but low in *Bacillus, Halolactobacillus,* and *Aerococcus*, indicating that the initial inoculation of Y12 had a positive effect on safety. The other two clusters of treatments were a combination of replicate without clear meaning. To emphasize that *Lactobacillus,* the critical genera for the Spanish-style olive fermentation, had an only outstanding presence in one replicate of T2 (inoculated with Lp13) and another of T4 (Y12). The consideration of the microbiota genetic information as CoDa allowed new insights on the table olive microbiota study.

3.6. Sensory analysis

The results of the ANOVA analysis (see Table S3 in supplementary material) showed that only brown spots descriptor discriminated among the different treatments, although the medians for crunch and bitterness were also significant. The effect of the panellist was always significant, although this behaviour is habitual and does not cause any trouble for the interpretation (their random influences are balanced in the design model). To notice that panellists were reliable and scoring similarly over sessions (non-significant effect of the session) while diverse interactions were reasonably low. Using Hotelling T2 test (Table 2) there were

significant overall differences among T1 (LPG1), T2 (Lp13), and T5 (sequential use of yeast and LAB starters), but not with respect to T6 (spontaneous) and T2 (Lp13) or T4 (Y12), although only T4 and T5 were significantly different from the spontaneous fermentation (T6).

When characterizing the samples from the different treatments (Table 3), it is observed the absence of fermentation defects (First or Extra Quality) since their values were always below the limit (<3) established in the COI method (2011). Apart from the significant lowest crunchiness and brown spot scores given to T3 and T6 treatments, respectively, and the highest bitterness and brown spots detected in T5, the other descriptors and overall acceptability had statically similar scores in all treatments. To detect some possible trends, the data were subjected to multivariate statistics. The PCA (Figure 8A) disclosed some interesting features. Treatments were grouped into 3 differentiated and meaningful clusters, indicating that T6 (spontaneous) and T1 show similar sensory trends; that is, LPG1 hardly disturbed the traditional fermentation process which, in turn, was associated with overall acceptability. T2 (Lp13) and T3 (Lpl15) were also similar and received good overall acceptability scores but were linked to some slight defects or products slightly different from the usual green olives (T6). Finally, T4 (Y12) and T5 (Y12+LAB) appeared strongly related among them, mainly related to bitterness browning or brown spot, but segregated from the other treatments. Bicluster analysis confirmed the precedent association and their relationships with descriptors (Figure 8B). Cluster 3 (T1 and T6) have the best characteristics since have low (in green) browning and brown spots scores while neutral (average, in black) in the rest of descriptors. Cluster 2 (T2 and T3) is associated with low bitterness and neutral (average, black) acceptability or hardness. Finally, Cluster 1 (T4 and T5) are characterized by their neutral to high bitterness or browning, but high scores in brown spots and low fermentation defects. Bicluster then characterizes treatments easily.

All treatments received overall R_{index} scores above 50%, but the order of preference was not parallel to the overall acceptability. T4 (Y12) was the most preferred concerning the control (61.2%), followed by T2 (Lp13) and T3 (Lp115) with 58.8%. However, T1 (LPG1) and T5 (sequential use of yeast and the combination of LAB starter) were considered rather similar to the spontaneous (preference of 52.4 and 54.8%, respectively). To notice that all treatments received greater appreciation than the traditional process but without significant differences among them.

4. Discussion

In this work, *Lactobacillus* and yeast strains with multifunctional properties, isolated from table olive biofilms, were used as starter cultures for the elaboration of Spanish-style table olives. Their final imposition frequency, acidification performance, production of AI-2, ability to colonize the surface of the fruit (to convert the olives in a "vehicle" of beneficial microorganisms to consumers), sensory evaluation, and influence on bacterial biodiversity, were determined as indicators of their suitability.

Information on how inoculum can affect the microbiota in Spanish style green table olive biofilms is still scarce. Omic approaches represent a good option, providing a deeper knowledge of the total biodiversity present in a food matrix. Recently, Cocolin et al. (2013), De Angelis et al. (2015), Medina et al., (2016), and Arroyo-López et al., (2016) used metataxonomic techniques to study the evolution of bacterial and fungus diversity during table olive fermentations. However, this work applies for the first time CoDa for the study and comparison of NGS data in a food environment. Its use in this type of data would not be optional (Gloor et al., 2017). The study revealed that, in spite of the inoculation and the development of lactic

fermentation, multiple bacterial genera could also be present through the fermentation. *Lactobacillus, Marinilactibacillus, Alkalibacterium,* and *Halolactobacillus* were the predominant genera observed at the end of the fermentative process in biofilms, albeit their frequencies could be modulated by the inocula. *L. plantarum* Lpl15 was the least effective inoculum to control the development of spontaneous microorganisms due to the sequences of *Aerococcus* genus in high proportion in T3 (Lp115) as corroborated by the CoDA analysis. This genus, currently containing five potential pathogens species was first identified by Willians et al. (1953) from samples of air and dust. However, scarce information of its presence in foods has been previously reported. Peirson et al. (2003) suggest that some *Aerococci* could be responsible for the greening of cocked meats product. Jeff-Agboola (2007) reported the presence of this genus in natural fermentation of African yam bean (*Sphenostylis sternocarpa* Harms) seeds for the production of otiru. No previous information about the presence of this genus in table olive processing has been found. The *Marinilactibacillus* genus has been reported by several authors during table olive processing (Lucena-Padrós et al., 2015; Lucena-Padrós and Ruiz-Barba, 2016; Rodríguez-Gómez et al., 2017). At present, the genus of *Marinilactibacillus* comprises only two species, *Marinilactibacillus piezotolerans* from deep subseafloor sediment of the Nankai Trough (Toffin et al. 2005), and *Marinilactibacillus psychrotolerans* from dead and living marine organisms in temperate and subtropical areas of Japan (Ishikawa et al. 2003). *Marinilactibacillus* spp. has been reported to ferment a wide range of organic substrates to produce lactic acid as the main end product of metabolism (Toffin et al. 2005). The genus *Alkalibacterium* is a halophilic and alkaliphilic bacteria reported for the first time by Ntougias and Russell (2001) as *Alkalibacterium olivoapovliticus*. This genus also metabolizes carbohydrates by lactic acid fermentation and is commonly part of the microbiota present during green olive fermentation.

Lucena-Padrós and Ruiz-Barba (2016) reported the presence of *Alkalibacterium* and *Halolactobacillus* genera during Spanish-style green table olive fermentations through PCR-DGGE techniques. Its origin could be related to the salt used for the preparation of brines.

The application of starter cultures in the present study led to a good acidification process and proper fermentation, preventing the appearance of pathogenic or spoilage microorganisms as confirmed by metataxonomic analysis, as well as the absence of organoleptic defects as deduced from the sensory evaluation. However, several authors have reported the inherent risks of spoilage in spontaneous olive fermentation (De Castro et al., 2002; Garrido-Fernández et al., 1997; Sánchez et al., 2001; Lanza, 2013). In this survey, the inoculation of the yeast in a first stage and later sequential inoculation with LAB strains was the most effective treatment, reaching the lowest pH values and the highest titratable acidity, especially during the first weeks after brining when the development of non-desirable microorganisms is more probable. Treatments inoculated exclusively with *L. pentosus* LPG1 and Lp13 were also more effective than the control treatment but the single inoculation with the yeast led to minor acidification. The high technological capacity of *L. pentosus* LPG1 and *L. pentosus* Lp13 was corroborated by molecular genotyping since 100% of the DNA profiles from the T1 and T3 isolates corresponded to these strains. Furthermore, Lp13 strain also was a good colonizer and biofilm former, as the 100% of the profiles analyzed in the rest of the treatment, including the control, showed a high similarity with its DNA profile. This strain also exhibited the highest production of lactic acid as measured by HPLC.

Currently, there is a trend to look for table olive starter cultures with not only technological characteristics but also with probiotic properties, but all previous studies were

carried out exclusively with microorganisms isolated from olive brines (Argyri et al., 2013; Bautista-Gallego et al., 2013; Botta et al., 2014; Peres et al., 2014). This work evaluated the technological performance of yeast and LAB strains isolated from the fruit surface, corroborating their ability to form biofilms during Spanish-style green table olive processing by SEM observation. The ability of these microorganisms to modulate the volatile composition of Spanish-style green table olive fermentations was also evaluated in previous studies (Benítez-Cabello et al., 2019b). Results showed that inoculation with strain Lp13 reduced the formation of volatile compounds, but on the contrary, inoculation with yeast Y12 increased their concentrations with respect to the spontaneous process.

On the other hand, the presence of AI-2 in biofilms on the surface of olives was detected for the first time in this work. This molecule is a member of a family of signalling molecules named autoinducers that plays an important role in cell-cell communication (Bassler et al., 2006). First identified in the marine bacterium *V. harveyi,* AI-2 is produced and detected by many Gram-negative and Gram-positive bacteria. Due to this characteristic, it has been recognized as a universal signalling molecule (Sperandio et al., 2003). Our data have also revealed that the highest production of AI-2 was detected at the maximum LAB counts on the olive surface. Inoculation with only yeast species give the lowest values of AI-2 but, on the contrary, the highest values were obtained in the sequential yeast-LAB inoculum. Taking into account that AI-2 production leads to biofilm formation by LAB (Liu et al., 2018), overexpression of *luxS* promotes stress resistance and biofilm formation of *Lactobacillus paraplantarum* L-ZS9 by regulating the expression of multiple genes (Park et al., 2016). AI-2 properties of kimchi, an eastern dish made from fermented cabbage or other raw vegetables, are associated with LAB involved in its fermentation (Park et al., 2016). A possible explanation for

the formation of those microbial communities in the skin of the olives could be first promoted by the production of AI-2 by the inoculated LAB population. Perpetuini et al. (2016) have also proved that *enoA1*, *gpi* and *oba* genes are necessary in *L. pentosus* to form an organized biofilm on the olive skin.

Rodríguez-Gómez et al. (2013, 2014), evaluated the performance as starters during green Spanish-style fermentations of diverse *L. pentosus* strains with promising results. However, Cocolin et al. (2013) showed, using molecular techniques, that genotypes of *L. plantarum* are predominant during Italian table olive fermentations. Results obtained in this work are in agreement with this finding since *L. pentosus* inocula were a better starter than *L. plantarum* for the fermentation of Spanish-style green table olives. Other authors have also studied the multifunctional properties of bacteria (Bevilacqua et al., 2010, De Bellis et al., 2010) and yeasts (Bonatsou et al., 2015, 2018; Porru et al., 2018; Oliveira et al., 2017) for their application as starter cultures. Tsapatsaris and Kotzekidou (2004), Segovia Bravo et al. (2007), and Arroyo-López et al. (2012a) have described the technological benefits of the use of yeasts for co-inoculation with LAB, reporting that they improved the growth of LAB and, consequently, an increased in the production of titratable acidity and aromas during fermentation (Sabatini et al. 2008). The results from this work are in accordance with these reports since the treatment inoculated with yeast and LAB inocula showed the best physico-chemical evolution, growth of LAB population and production of AI-2, and also was differentiated by the panellists. Moreover, in the specific case of yeast Y12, it can also offer other remarkable technological features such as improvement of the organoleptic profile of fermentation by production of volatile compounds (Benítez-Cabello et al., 2019b) and biological debittering of fruits due to their esterase and β-glucosidase activities (Rodríguez-Gómez et al., 2012a). Recently, Sidari et al. (2019) reported *W.*

anomalus as one of the most important species isolated from naturally fermented *Nocellara messinese* table olives, with relevant technological applications such as β-glucosidase and catalase activities.

Regarding organoleptic characteristics, notice that all treatments lead to fermented products classified as First or Extra quality according to IOC evaluation criterion (IOC, 2011), absence of fermentation defects. Nevertheless, the use of different starters can influence other attributes. Among those included in this evaluation sheet, the medians of crunchiness and bitterness were significant but had not discriminant power, which only was found for brown, attribute which cannot necessarily be related to the effect of the starters. Interestingly, the overall acceptability was scored as similar for all treatments and, therefore, their use will not modify the traditional expected sensory profile. However, T4 (Y12), T2 (Lp13), and T3 (Lpl15) were slightly preferred when evaluated according to the R_{index} test (about 60% preference). This contrast with other experiments (Alfonzo et al., 2018) which reported worse satisfaction for some treatments inoculated with *L. pentosus* than for the control which was, in turn, similar to some others trials processed in different conditions but, usually, such differences can hardly be demonstrated. In a study on the sensory assessment by consumers of traditional and potential probiotic green Spanish-style olives inoculated with *L. pentosus* TOMC-LAB2, a first approach led to the conclusion that both products were perceived as similar, being necessary a biplot, based on Canonical Variate Analysis, to disclose differences between them in salty and overall scores. In our work, differentiated clusters were also only achieved after PCA analysis or biclustering, showing that T6 (spontaneous) and T1 (LPG1) were similarly perceived by the panel but different from T2 (Lp13), and T3 (Lp115) which were considered slightly different to the traditional product; in addition, the incorporation of the yeast Y12 to the inoculum (T4 and

T5) also led to particular characteristics, mainly associated to the absence of fermentation defects but possible lower debittering properties. Differences between these clusters regarding volatile compositions were also demonstrated (Benitez Cabello, 2019b). Hence, our results are in agreement to the conclusion that a revision of the technological procedures may improve fermentation performance and the final quality of table olives (Aponte et al., 2010).

4. Conclusions

The inoculation with autochthonous strains isolated from olive biofilms proved to be an effective practice for improving the fermentative process of Spanish-style green table olives. The best physico-chemical results were obtained after inoculation with the yeast Y12 followed by a combination of the LAB strains as reflected by the more rapid pH drop, higher acid production, sugar consumption, LAB growth, biofilm formation, and production of AI-2, especially detected during the first weeks after brining. Therefore, *L. pentosus* LPG1 and *L. pentosus* Lp13 alone or in combination with Y12 have proved to be good candidates for the production of functional olives. Metataxonomic analysis at the end of the fermentation also showed the presence in a high proportion of other bacterial genera in addition to *Lactobacillus*, but the role played by these microorganisms in the fermentation process requires further investigation.

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Figure Legends

Figure 1. Evolution of the main physico-chemical parameters during the different treatments assayed. T1 stands for processes inoculated with LPG1; T2 for Lp13; T3 for Lpl15; T4 for yeast Y12; T5, for Y12 followed by a combination of LPG1+Lp13+Lpl15), and T6, for the spontaneous process. Error bars denote standard deviation calculated from duplicate fermentation vessels.

Figure 2. Changes in the lactic acid bacteria and yeast populations in the cover brines (a, b) and fruits epidermis (c, d) throughout fermentation. T1 stands for treatments inoculated with LPG1; T2 for Lp13; T3 for Lpl15; T4 for yeast Y12; T5, for Y12 followed by a combination of LPG1+Lp13+Lpl15; and T6, for the spontaneous process. Error bars denote standard deviation calculated from duplicate fermentation vessels.

Figure 3. AI-2 presence, measured as counts per second (CPS), in biofilms through fermentation. T1 stands for treatments inoculated with LPG1; T2 for Lp13; T3 for Lpl15; T4 for yeast Y12; T5, for Y12 followed by a combination of LPG1+Lp13+Lpl15; and T6, for the spontaneous process. Error bars denote standard deviation calculated from duplicate fermentation vessels.

Figure 4. SEM of the fermenting olive surface showing biofilm formation between yeast and LAB after 35 days of fermentation.

Figure 5. Dendrogram for the LAB isolates obtained from biofilms at the end of the fermentation (65 days), using rep-PCR analysis and according to treatments. Fingerprinting profiles of inocula LPG1, Lp13, and Lpl15 are marked with (*).

Figure 6. Frequency of sequences obtained by metataxonomic analysis for the different bacterial groups according to treatments. T1 stands for processes inoculated with LPG1; T2 for Lp13; T3 for Lpl15; T4 for yeast Y12; T5, for Y12 followed by a combination of LPG1+Lp13+Lpl15; and T6, for the spontaneous process. Average values were obtained from duplicated experiments.

Figure 7. Covariance biplot (A) and bicluster (B) graphs obtained from Co-Da metataxonomic analysis at the end of the fermentation process. T1 stands for treatments inoculated with LPG1; T2 for Lp13; T3 for Lpl15; T4 for yeast Y12; T5, for Y12 followed by a combination of LPG1+Lp13+Lpl15; and T6, for the spontaneous process. The variance of the log-ratio between two parts (variables) is proportional to the length of the corresponding link between their arrow ends.

Figure 8. Application of PCA (A) and bicluster (B) analysis to the sensory data obtained from the different treatments, after packaging and equilibrium. T1 stands for treatments inoculated with LPG1; T2 for Lp13; T3 for Lpl15; T4 for yeast Y12; T5, for Y12 followed of a combination of PG1+Lp13+Lpl15; and T6, for the spontaneous process.

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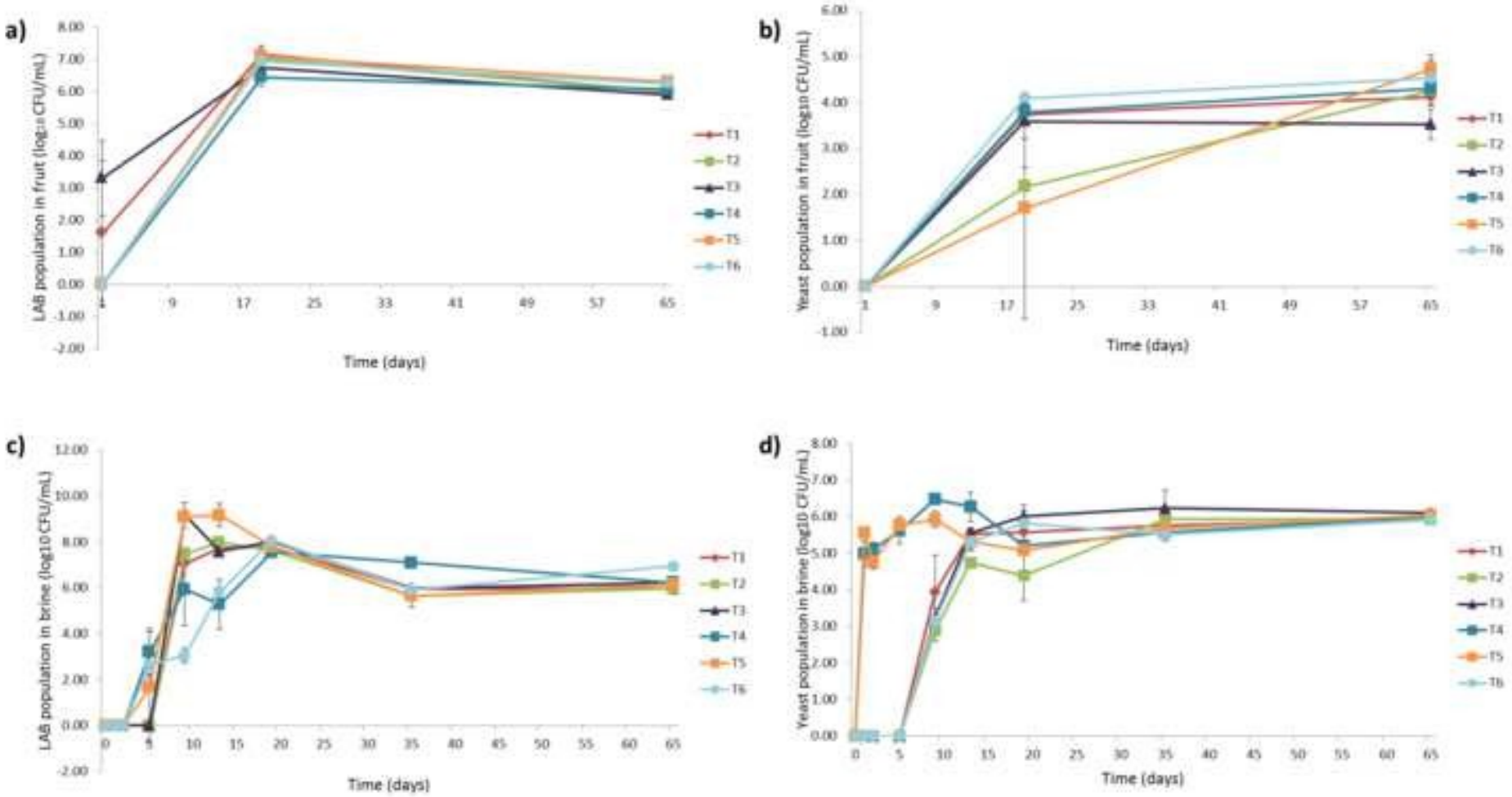

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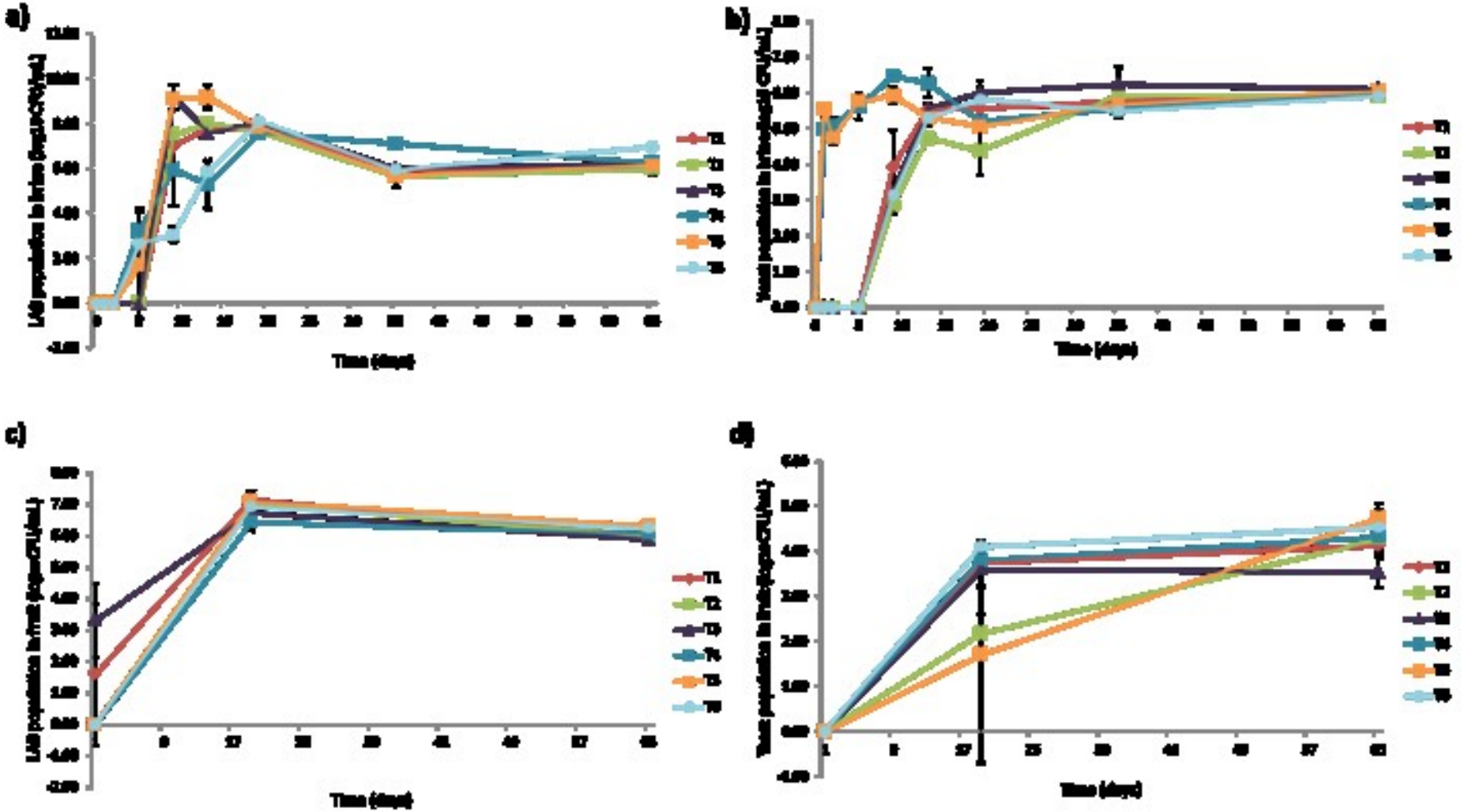

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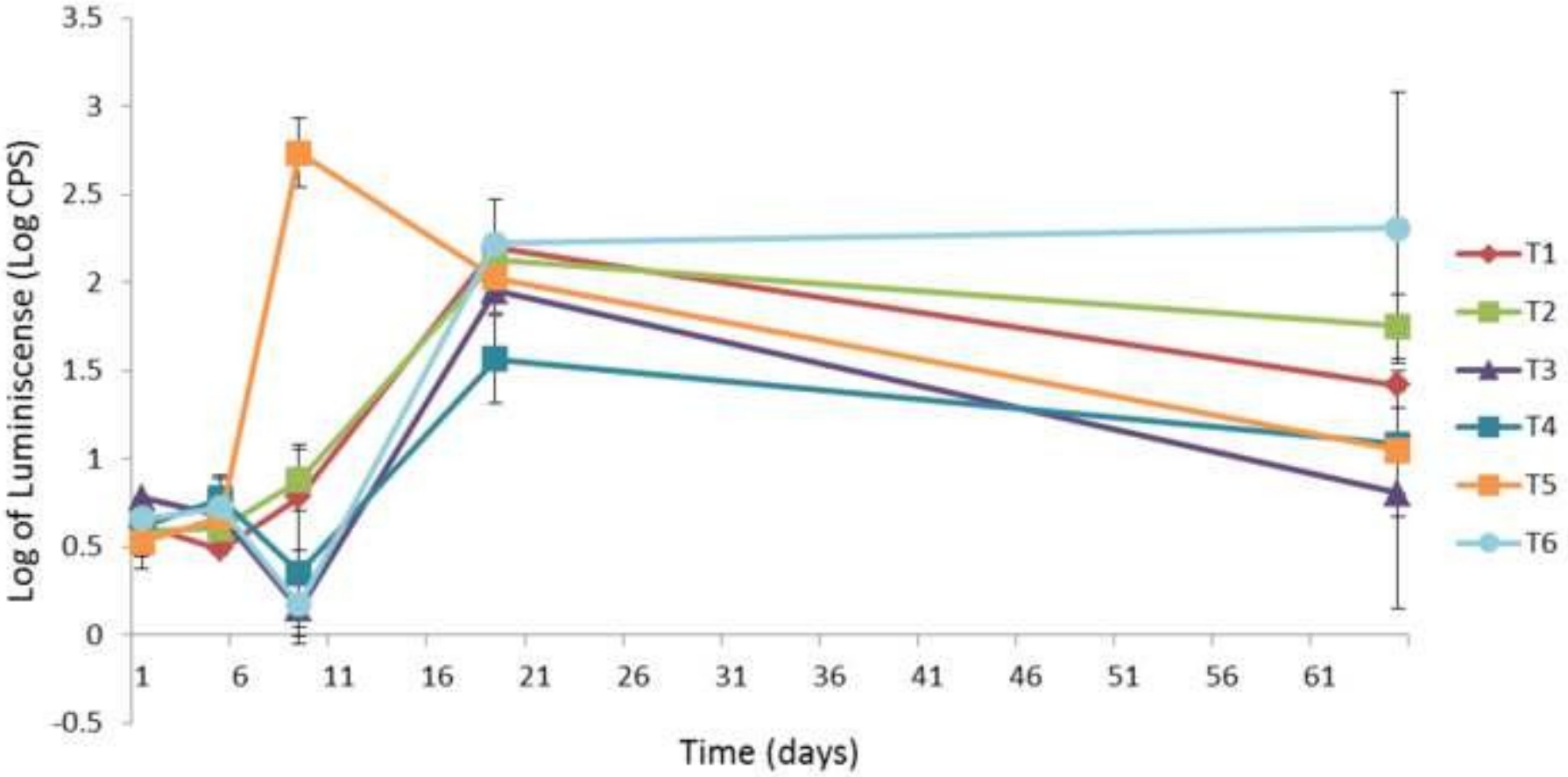

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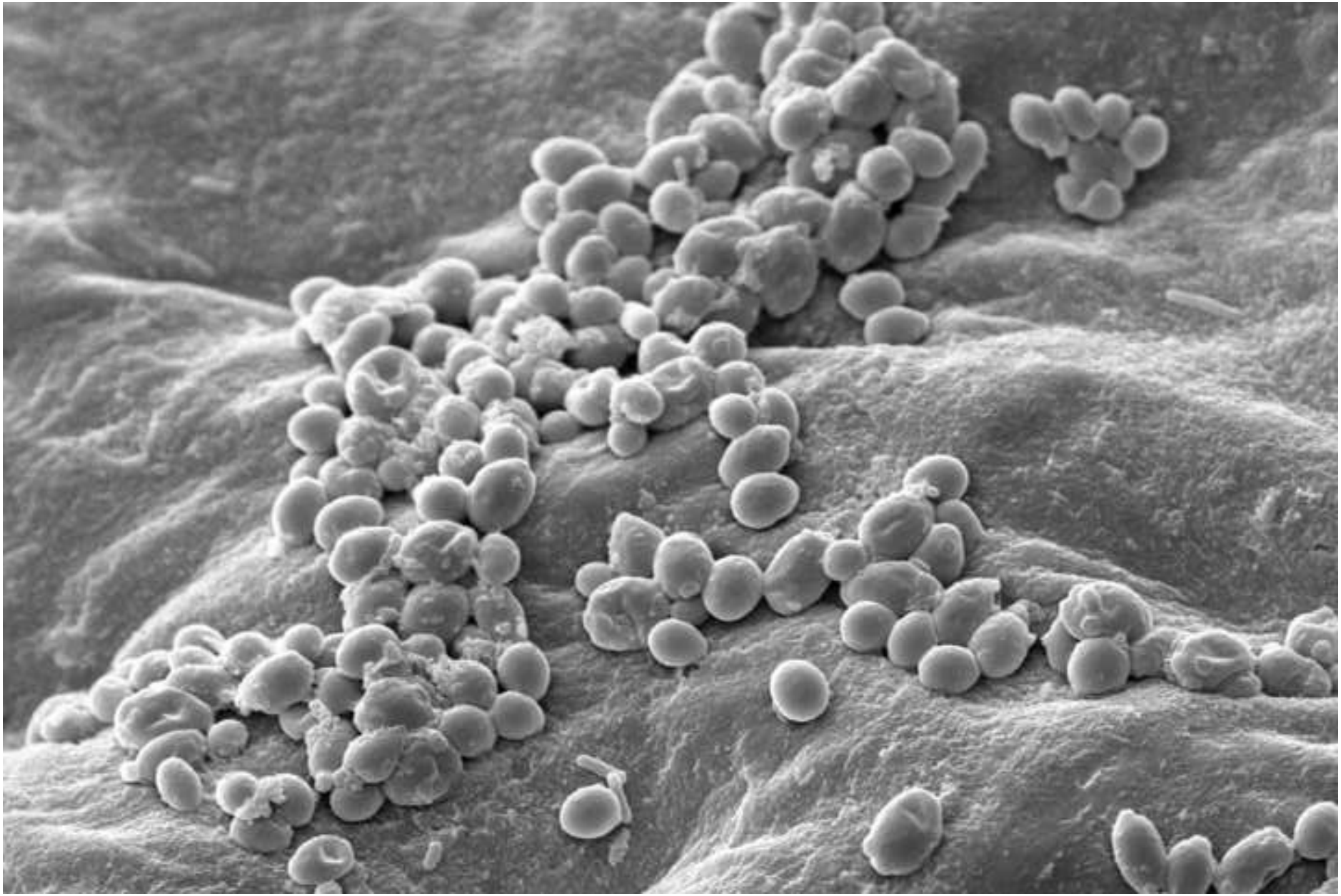

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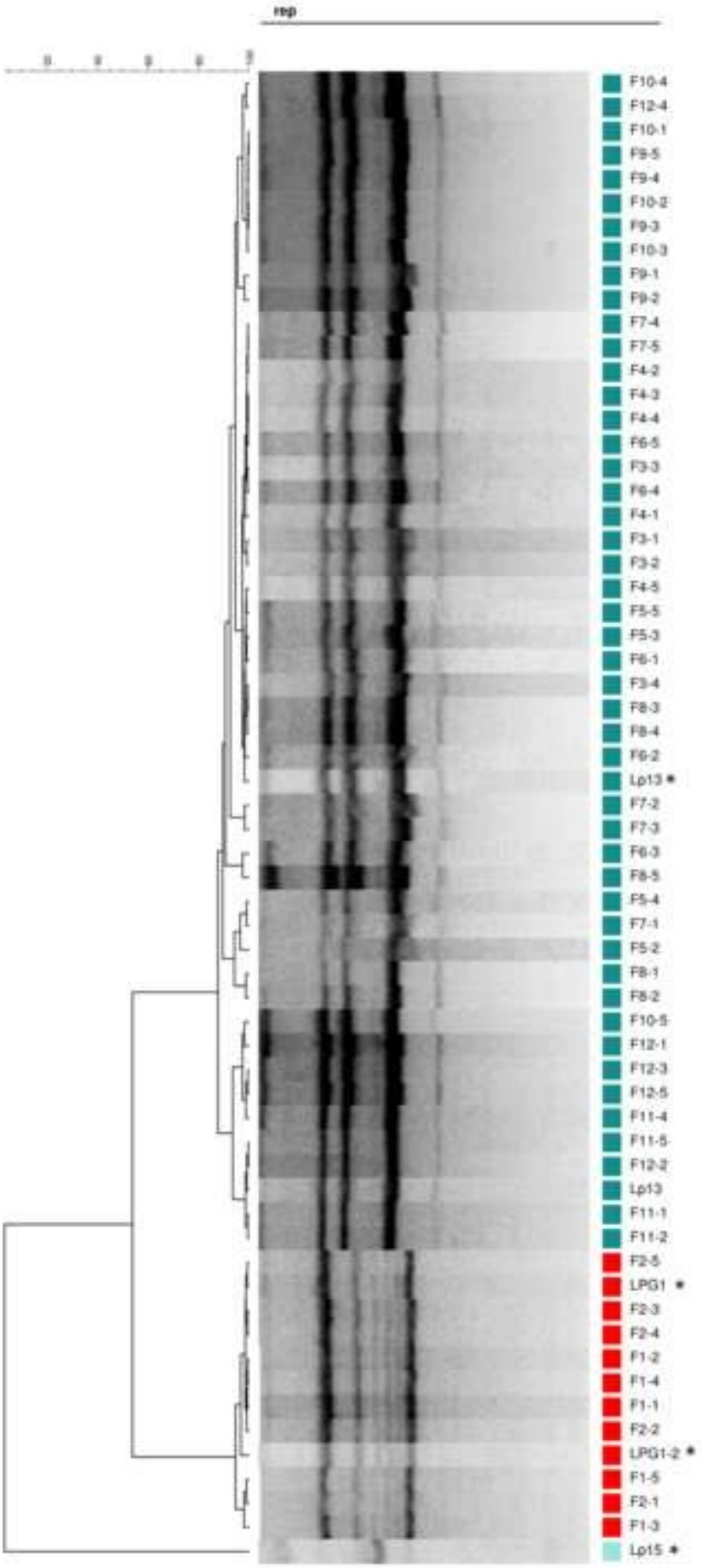

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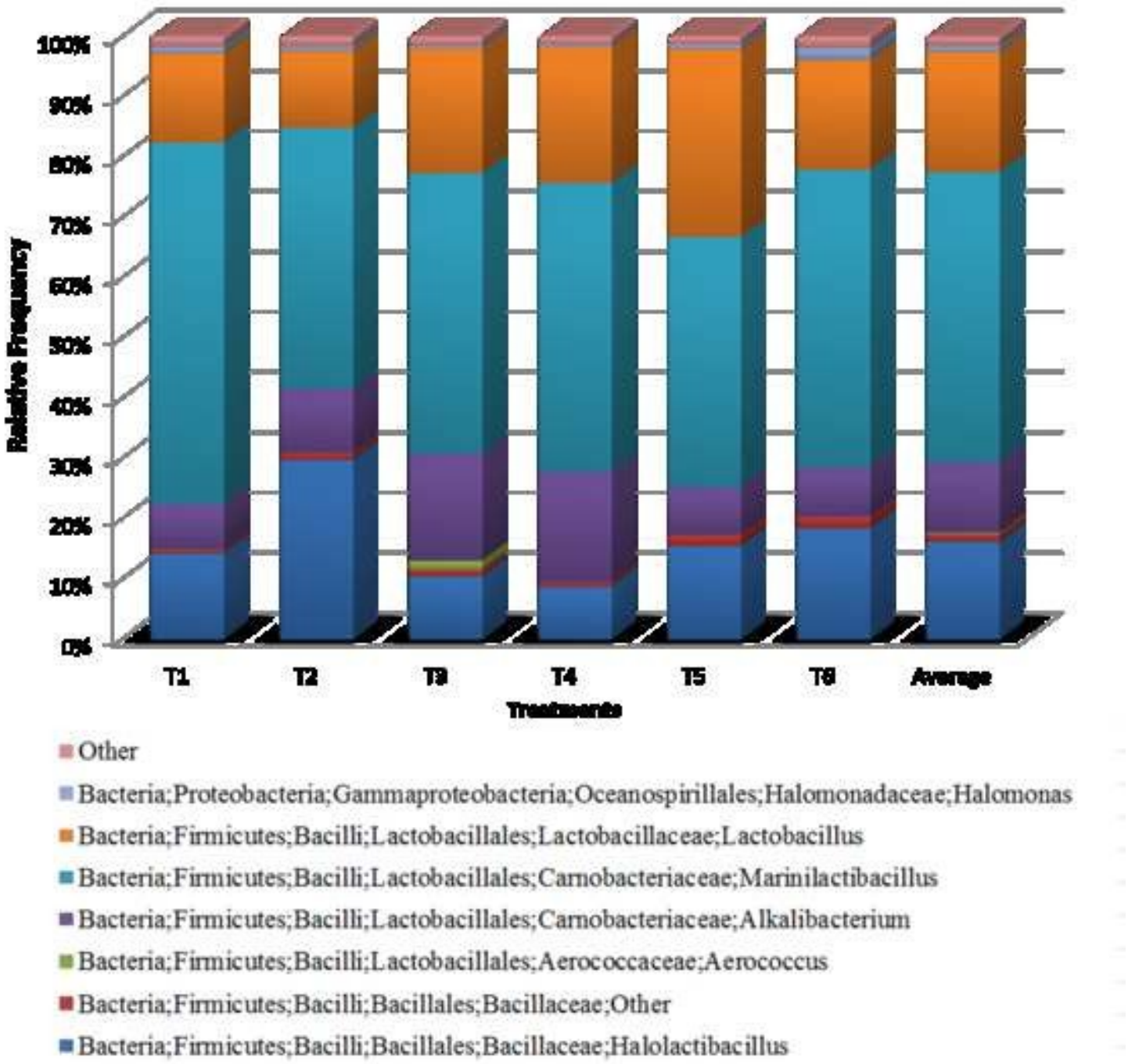

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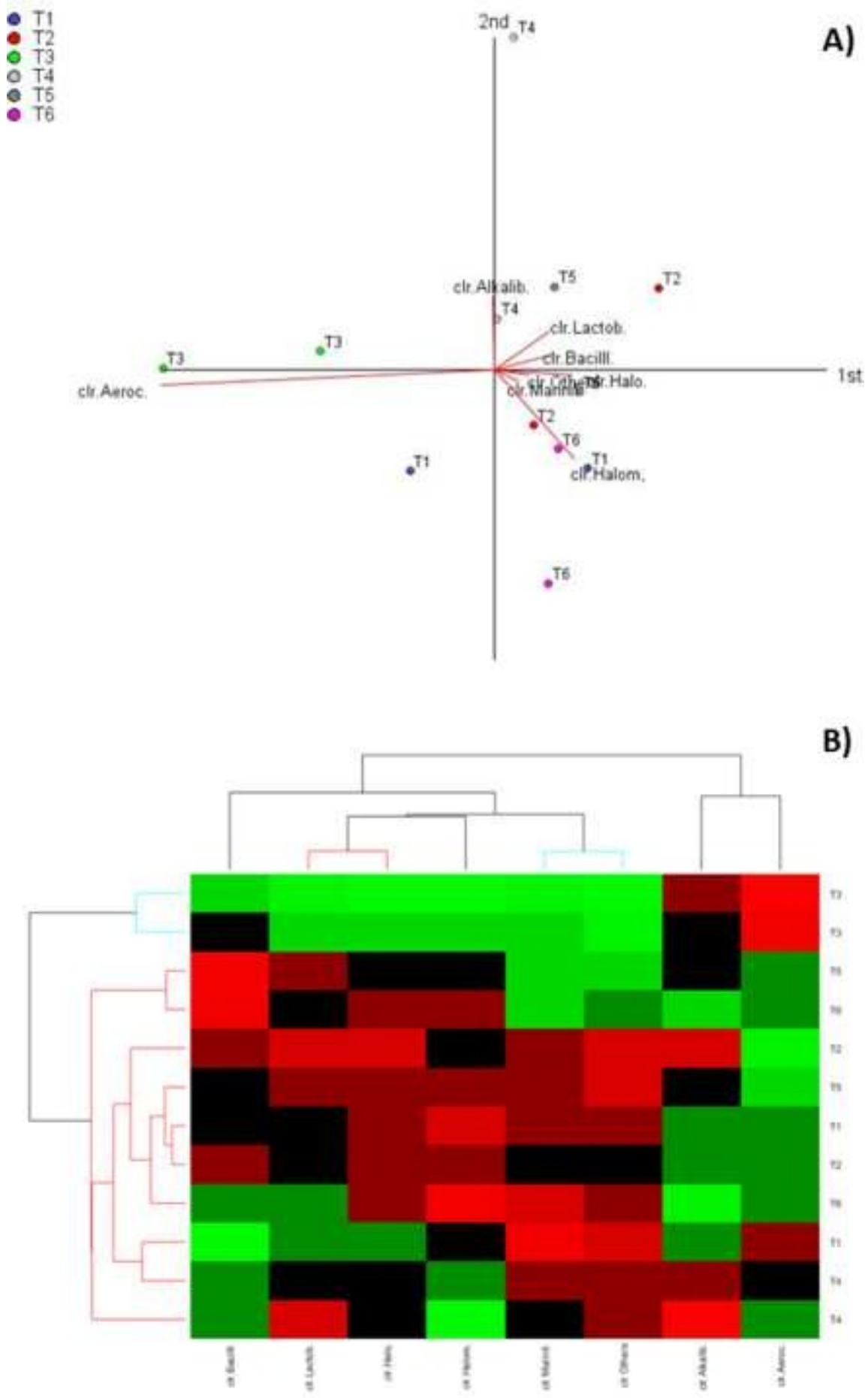

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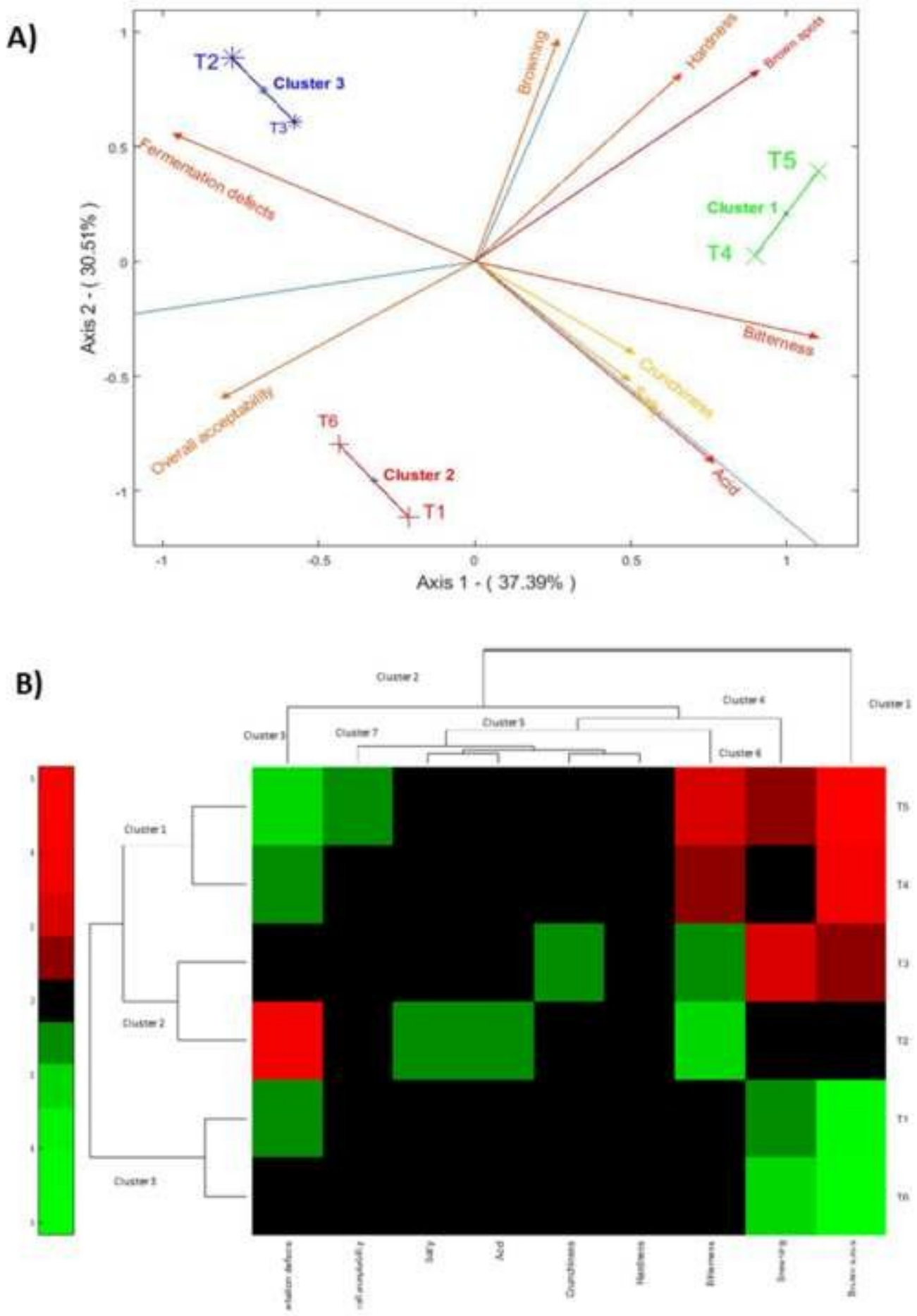

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Conflicts of Interest: The authors declare no conflict of interest.

Data in Brief