LWT - Food Science and Technology 166 (2022) 113787

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Optimisation of working parameters for lactic acid bacteria and yeast recovery from table olive biofilms, preserving fruit integrity and reducing chloroplast recovery

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ARTICLE INFO

Keywords:
Biofilms
Lactic acid bacteria
Metataxonomic analysis
RSM
Table olives

ABSTRACT

The study of the table olive biofilms is usually achieved using a stomacher which causes the physical rupture of pitted olives in an osmotic media but does not differentiate between those on the surface and inside the fruits. Besides, the high amount of eukaryotic DNA released from the vegetable cells makes the further molecular study of the detached microbiota complex. This work applies Response Surface Methodology, based on a D-optimal experimental design and glass beads, to recover lactic acid bacteria (LAB) and yeasts from the biofilm of fermented olives without crushing the flesh. The RSM showed that the best simultaneous results were obtained by shelling the olives, at 8 °C, with glass beads (6 mm diameter) in a 0.16 (w/w) bead/olive ratio for 15 min at 330–400 rpm. Its application recovered lower LAB (possibly because of excluding those in the flesh), but similar yeast counts to the stomacher. The metataxonomic analysis of total DNA from biofilms showed that the glass bead-based protocol reduced the chloroplast sequences and improved the genera assignation for bacteria (16S) and yeast (ITS). The new non-destructive procedure enhances biofilm studies and may allow validating table olives as a carrier of probiotic microorganisms.

1. Introduction

Table olives are fermented fruits characteristic of the Mediterranean basin, with Spain, Egypt, and Turkey as the leading producers (IOC, 2020). The main commercial presentation is green Spanish-style table olives, debittered with alkaline lye and brined in a 10% NaCl solution. Lactic acid bacteria (LAB) and yeasts are the main microorganisms present during this product's fermentation, storage, and packaging, determining its final flavour, quality, and safety (Garrido-Fernández et al., 1997). However, other types of bacteria such as *Enterobacteriaceae, Propionibacterium,* or *Clostridium* spp might also be present (Arroyo-López et al., 2008, 2012; Hurtado et al., 2012).

A biofilm is defined as a consortium of microorganisms embedded in an extracellular polymeric substance (EPS) produced by themselves that firmly adheres to a solid (abiotic or biotic) surface (Costerton, 2007; Davey & O'toole, 2000; Remis et al., 2010). Recently, several studies demonstrated that microorganisms from the fermentation brine could colonise the olive surface, forming polymicrobial communities (biofilms) attached to the epidermis of the fruits (Arroyo López et al., 2012; Domínguez-Manzano et al., 2012; Lavermicocca et al., 2005; Nychas et al., 2002). The LAB adhesion mechanisms to form biofilms are diverse but always require a correlation between the hydrophilicity surface of the bacterial cells and a hydrophilic surface (Faten et al., 2016). Microbial structures, such as fimbriae, flagella, and pili, play an essential role in the biofilm formation and EPS production, but environmental factors, such as pH, temperature, exposure time, and ionic forces, can also affect the adhesion (Domínguez-Manzano et al., 2012; Jahid & Ha, 2012; Wimpenny, 2009). The biofilms formed on the olive surface usually include LAB (mainly *Lactiplantibaillus pentosus* and *Lactiplantibacillus plantarum*) and yeast species (*Wickerhamomyces anomalous, Saccharomyces cerevisiae*, among others). The microorganisms released from the olive biofilms may reach up to 10 million CFU/g (Arroyo López et al., 2012; Domínguez-Manzano et al., 2012; Grounta & Panagou, 2014). Initially, these biofilms can also harbour *Enterobacteriaceae*, which disappear gradually as the process advances and the pH is progressively lower (Rodríguez-Gómez et al., 2014).

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https://doi.org/10.1016/j.lwt.2022.113787
Received 14 February 2022; Received in revised form 10 June 2022; Accepted 17 July 2022
Available online 20 July 2022
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The microorganisms in the olive biofilms are not released by simply washing the olive surface. For quantification, they should be detached by physical (homogenisers, disintegrators, or glass beads) (Aponte et al., 2010; Coton et al., 2006; Pereira et al., 2008; Perpetuini et al., 2016) or chemical methods (enzyme cocktails) (Arroyo López et al., 2012; Böckelmannet al., 2003; Domínguez-Manzano et al., 2012). Benitez-Cabello et al. (2015) recently compared several extraction mechanisms to recover both LAB and yeast from fermented table olives. The mechanical extraction with a stomacher was an appropriate methodology, but its application also released eukaryotic DNA from ruptured vegetable cells, a severe drawback for further molecular studies; besides, it does not segregate between microorganisms on the olive surface or from inside the flesh. However, reliable microbial counts in the olive biofilm are essential for characterising this product as a carrier of probiotic microorganisms.

Shelling fermented olives with glass beads could be an alternative non-destructive method to count only the microbial population adhered to the olive surface. Several factors, such as glass bead size, time, and glass bead/olive ratio, may influence the removal, but their effects are unknown. Response Surface Methodology (RSM) could be an appropriate tool for investigating the simultaneous impact of these variables and optimising the bacterial and yeast recovery (Myers et al., 2016). RSM was successfully applied to ferment Manzanilla-Aloreña in a mixture of nutrient salts (Bautista-Gallego et al., 2010) and Gordal table olives (Bautista-Gallego et al., 2011). D-optimal design is widely chosen to apply the RSM methodology (Myers & Montgomery, 2002) and could help improve LAB and yeasts recovery from table olive biofilms.

This work aims to develop a non-destructive procedure for detaching microorganisms from table olive biofilms while reducing the mitochondrial DNA release and improving the subsequent metagenomics analysis of the involved microorganisms.

2. Material and methods

2.1. Olive samples

Green Manzanilla cultivar olives, obtained in the 2020/2021 season and provided by JOLCA S.L. (Huevar, Sevilla), were treated with a 2.0% (w/vol, as usual) NaOH solution until 2/3 of the pulp, soaked in tap water for 3 h, and distributed into 250 mL glass containers (180 gr fruits + 136 mL brine). The fruits were covered with 11% NaCl brine, the containers closed, pasteurised at 80 °C for 10 min, and stored at 4 °C for 7 days. When pH was around 8.0 and salt 5%, the containers were tempered, and most of them inoculated to reach 5.0 $\log_{10}$ CFU/mL for both LAB and yeasts in the brine while the rest were devoted to checking the pasteurisation process. The inocula were 50% OleicaStarter Advance (a mix of 3 strains of *Lactiplantibacillus pentosus* species) and 50% OleicaYeasts (a blend of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae*) specially designed for table olives (TAFIQS in Foods S.L, Seville, Spain). The physicochemical (pH, salt, and titratable acidity) and microbiological (LAB and yeasts) characteristics of all containers were checked at the 2, 6, 15, 21 and 24 days of fermentation, following the methodology suggested by Garrido-Fernández et al. (1997).

2.2. Experimental design

The variables supposed to influence the LAB and yeasts detachment and their ranges (in brackets) were the time of agitation (5–15 min), shaking (200–400 rpm), beads/pitted olives ratio (0.16–0.50 w/w), and size of glass beads (considered as a qualitative factor with three levels: 2, 4, and 6 mm diameter). The RS was developed through a D-optimal experimental design with 2 replicates to estimate the pure error. The responses were the released LAB, and yeast ($\log_{10}$ CFU/g) counts. The specific combination of variable levels for each run (treatment) is detailed in Table 1. Optimal designs are straight optimisations based on an optimality criterion and the model that will be fit. The optimality criterion used in generating D-optimal design is one of maximising |X'X|, the determinant of the information matrix. In the design of experiments, optimal designs allow estimating the model's parameters with a minimum variance (Design Expert v 12, Stat-Easy, Inc. MN, USA).

Table 1
D-optimal design used for the optimisation of the simultaneous LAB and yeast recovery by the glass beads (non-destructive) protocol. It also includes the responses expressed as $\log_{10}$ CFU/g and the results of applying the stomacher protocol to the same olives (St 1 and St 2 rows).

Run number	A - Time (min)	B - Agitation (rpm)	C- glass beads/ olive ratio	D- glass bead size (mm)	LAB ($\log_{10}$ CFU/g)	Yeast ($\log_{10}$ CFU/g)
1	10.32	298.35	0.33	6	7.34	5.68
2	5.00	365.47	0.50	4	7.50	5.65
3	5.00	200.00	0.50	2	7.28	5.50
4	9.25	400.00	0.50	2	7.37	5.42
5	15.00	200.00	0.32	2	7.42	5.59
6	15.00	306.98	0.50	2	7.38	5.52
7	11.25	339.33	0.19	2	7.41	5.41
8*	15.00	235.80	0.16	4	7.28	5.50
9	5.00	400.00	0.16	2	7.15	5.35
10	12.53	200.00	0.50	4	7.31	5.40
11	15.00	400.00	0.50	6	7.64	5.57
12	7.43	400.00	0.16	4	7.33	5.39
13*	15.00	235.80	0.16	4	7.35	5.59
14	5.00	200.00	0.50	6	7.60	5.51
15	15.00	400.00	0.44	4	7.34	5.49
16	5.00	200.00	0.22	4	7.15	5.11
17	5.00	200.00	0.50	6	7.30	5.26
18	9.12	200.00	0.16	2	7.47	5.51
19	6.93	250.00	0.41	4	7.27	5.51
20	5.47	270.76	0.16	6	7.11	5.50
21	15.00	200.00	0.16	6	7.66	5.52
22	5.00	400.00	0.16	6	7.41	5.58
23	15.00	400.00	0.16	6	7.62	5.76
24	5.00	326.37	0.36	2	7.36	5.35
St 1	NA	NA	NA	NA	7.64	4.39
St 2	NA	NA	NA	NA	7.68	4.53

Notes: Levels 1, 2, and 3 correspond to glass bead sizes (2, 4, and 6 mm of diameter, respectively. * replicates.

The analysis of the results led to estimating the RS equation and finding the optimal conditions. Later, they were applied to olives from another similar independent fermentation process, and the LAB and yeast recovered (expressed as CFU/g and $\log_{10}$ CFU/g) compared with those from stomacher.

2.3. Biofilm detachment

When the LAB and yeast populations in the inoculated containers reached the highest values (at the 24th fermentation day, according to Arroyo López et al., 2012; Grounta & Panagou, 2014), their olives were subjected to the biofilm detachment.

The extraction with the stomacher (a destructive method) followed the protocol described by Benítez-Cabello et al. (2015). Briefly, fruits (10 g) were washed twice with a 0.9% sterile NaCl solution, pitted, weighed in sterile conditions, and transferred into a stomacher bag containing 25 mL of sterile saline solution. Then, the fruits were homogenised for 3 min at 300 rpm in a stomacher model Seward 400 (Seward, United Kingdom).

The glass bead (non-destructive) detachment followed the Perpetuini et al. (2016) protocol, with slight modifications. The whole fruits (10 g) were washed twice with 0.9% sterile saline solution and then transferred into a 50 mL falcon tube containing the glass beads of the size and proportion required by the design and 25 mL of a sterile saline solution. Then, the fruits and glass beads were shelled at 8 °C for the corresponding time and shaking intensity (see Table 1). After biofilm detachment, the fruits were pitted and weighed in sterile conditions. For LAB and yeast quantification, direct and suitable dilutions (10^{-2} and

10^{-4}) of the saline solutions containing the released microorganisms were plated using a spiral system (easySpiral Dilute, Interscience, France). For LAB, the solution was spread onto De Man, Rogosa, and Sharpe (MRS) agar medium (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 0.02% sodium azide (Sigma, St. Louis, MO, United States). For yeasts, the yeast-malt-peptone-glucose (YM) agar medium (Difco, Becton and Dickinson Company, Sparks, MD, USA), supplemented with oxytetracycline and gentamicin sulphate (0.005%, w/v) as selective agents, was used. The plates were incubated a 37 °C for 48 h for LAB and 30 °C for 48 h for yeasts in an incubator Selecta™ 2000207 (Barcelona, Spain). Colonies were counted using an automatic counter Interscience Scan4000 (Interscience, France) image analysis system. Finally, the counts were referred to the weight of olives and expressed as $\log_{10}$ CFU/g.

2.4. Metataxonomic analysis

The second fermentation was also used to study the influence of the glass beads and stomacher extraction systems on the quality of the metataxonomic analysis of the recovered biofilm. Its olives were subjected again to the two recovery protocols. For stomacher, the same conditions mentioned above were applied, while in the case of glass-bead detachment, several combinations around the optimal conditions deduced from the RS were used. The LAB and yeast counts in the resulting solutions were expressed as $\log_{10}$(CFU/g), and the data were analysed according to extraction methods.

For metataxonomic analysis, 15 mL of the saline solutions obtained from each biofilm extraction methodology was spun by duplicated at 9000×*g* for 15 min. The supernatant was then removed, and the pellet was washed twice with sterile saline solution before being stored at −80 °C until DNA extraction. The total genomic DNA from samples was extracted and purified according to the manufacturer's instructions, using the PowerFood Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA), and sent for sequencing to FISABIO (Valencia, Spain). Before sequencing, the purified DNA was measured using a Qubit fluorimeter (Thermo Fisher Scientific, Waltham, USA), always obtaining values above 0.2 ng/μL.

For the bacterial populations, the V3 and V4 regions (459 bp) of the 16S ribosomal RNA gene were amplified with the designed primers surrounding conserved regions (Klindworth et al., 2013) following the Illumina amplicon libraries protocol. DNA amplicon libraries were generated using a limited PCR cycle: initial denaturation at 95 °C for 3 min, followed by 25 cycles of annealing (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s), and a final extension at 72 °C for 5 min, using a KAPA HiFi HotStart ReadyMix (KK2602). Then, the Illumina sequencing adaptors and dual-index barcodes (Nextera XT index kit v2, FC-131-2001) were added to the amplicons.

For the fungi population, DNA samples were submitted to PCR amplification of the ITS1 region located inside the fungal nuclear ribosomal DNA (rDNA) with the designed primers surrounding conserved regions ITS1-F_KYO2 (18S SSU 1733–1753) and ITS2_KYO2 (5.8 2046–2029) (Gardes & Bruns, 1993; Toju et al., 2012). DNA amplicon libraries were generated using the following limited PCR cycle: initial denaturation at 95 °C for 3 min, followed by 28 cycles of annealing (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s), and a final extension at 72 °C 5 min, using a KAPA HiFiHotStart ReadyMix (KK2602).

Then, the Illumina sequencing adaptors and dual-index barcodes (Nex-tera XT index kit v2, FC-131-2001) were added to the amplicons in both cases. Libraries were normalised and pooled before sequencing. The pool containing indexed amplicons was loaded on the MiSeq reagent cartridge v3 (MS-102-3003) spiked with 25% PhiX control to improve base calling during sequencing, as recommended by Illumina for amplicon sequencing. Sequencing was conducted using a paired-end, 2 × 300 bp cycle run on an Illumina MiSeq sequencing system.

2.5. Statistical and bioinformatics analysis

The design was deduced and studied using Design Expert v 12.0 (Stat-Easy, Inc., MN, USA). First, a sequential model sum of squares (type II) is suggested as the most appropriate model for describing the results. Then, the RSM fit was studied by ANOVA, using the partial sum of squares (type III), the fit characteristics evaluated, and the variables in the model selected. Models were considered appropriate when significant at $p \leq 0.05$ and showed a non-significant lack of fit. The retained terms were those chosen by stepwise regression (p-values to enter and remove $p \leq 0.05$ and $p \leq 0.10$, respectively, to prevent the procedure get into an infinite loop) or required to maintain the model's hierarchical condition (necessary for predictions). Finally, numerical and graphical optimisations were performed.

The LAB and yeast counts from applying the two protocols to the second fermentation were compared by ANOVA, using XLSTAT v 2017 (Stat Soft, Paris, France).

Metataxonomics data were analysed using the R package phyloseq 1.32.0 (McMurdie & Holmes, 2013) under default parameters. For each sample, only the most abundant sequences (>0.1%) were retained as Amplicon Sequence Variant (ASV); the remaining reads were clustered against those ASVs allowing one mismatch to correct for error sequencing. Bacterial taxonomy was assigned using the SILVA 138 SSU database (containing 2,225,272 sequences) (Quast et al., 2012). The fungi taxonomy was assigned using the full UNITE + INSDC (containing 1,796,591 sequences) (Abarenkov et al., 2020). Finally, plots were generated using ggplot 2 3.3.2 (Wickham, 2016).

3. Results and discussion

3.1. Fermentation monitoring

Previous studies have reported that the LAB biofilm formation on green Spanish-style olives reaches its maximum population after 20–30 days of fermentation (Arroyo López et al., 2012; Grounta & Panagou, 2014). Thus, the physicochemical and microbiological analyses of brines (inoculated and sterile containers) were carried out for 24 days. The inoculated containers followed the typical evolution of the Spanish style but without *Enterobacteriaceae* presence (data not shown). On the contrary, no microbial growth was detected in the non-inoculated treatment, confirming the pasteurisation process's effectiveness. After the 24th day of fermentation, the LAB and yeast populations in brines reached the expected maximum (8.4 $\log_{10}$ and 6.4 $\log_{10}$ CFU/mL, respectively), and the values of pH, titratable acidity, and salt concentration in brine were 3.92, 0.62% acidity, and 4.9% NaCl, respectively. In contrast, in the non-inoculated (but pasteurised) treatments, the levels were 5.95, 0.1%, and 5.1%, indicating the absence of microbial activity. In inoculated Spanish-style table olives, Arroyo López et al. (2012) reported maximum values of around 8.5 and 6 $\log_{10}$ CFU/mL for LAB and yeasts, respectively, while Grounta and Panagou (2014) found 6.5–7 and 4.5 $\log_{10}$ CFU/mL in inoculated black olives.

3.2. Optimisation of the non-destructive protocol

Recently, Benítez-Cabello et al. (2015) reported the use of destructive (stomacher) and non-destructive procedures (enzymatic and ultrasound) methodologies for the recovery of the bacteria and yeasts adhered to table olive peel. The highest yields were achieved using the stomacher for 1 min (LAB) and sonication for 5 min (yeasts). Later, Perpetuini et al. (2016) used glass beads (a non-destructive procedure) to detach the bacteria adhered to olive peel, using 2 mm glass bead size, 200 rpm for 1 h, and 4 °C but did not analyse the variables involved in the removal. In this work, a D-optimal design and RSM were applied to study the effects of shaking intensity (agitation), time, glass bead sizes, and bead/fruit ratio on the simultaneous recovery of the LAB and yeasts populations attached to Spanish-style fermented olive. The responses

were the LAB and yeast released after applying, in random order, the combination of variables (runs or treatments) of the experimental design to inoculated-fermented olives (Table 1).

3.2.1. LAB recovery

For LAB recovery, the sequential sum of squares suggested a quadratic model in which the terms were selected by stepwise regression. The ANOVA showed that the chosen model was significant (p=0.0247, with only a 2.47% probability that it could be due to noise), and the lack of fit was not significant (Table 2, lactic acid bacteria). Regarding terms, time (p=0.0165) and glass bead size (p=0.0528) were considered significant (lower than the p-value to remove, $p \leq 0.10$). Besides, the interaction time·bead/olive ratio was retained because of its initial significant contribution and its p-value closeness to the removal limit. The precision was 6.411, which means an adequate signal/noise ratio to identify the effects, and the plot of the internally studentised residual showed a good agreement with the normal distribution. That is, the model for LAB recovery was adequate to navigate within the experimental region.

Then, LAB recovery was a function of the agitation time, the interaction time·glass bead/olive fruit ratio, and the glass bead size, with an equation for each level of the last one because of its qualitative character (Table 2, lactic acid bacteria). The functions, expressed in terms of actual factors, were:

$$\text{LAB } (\log_{10} \text{CFU/g}) = I + 0.036356\ A + 0.81236\ C - 0.065427\ A\ C. \quad \text{Eq 1}$$

Where I was +6.95428, +6.89036, and +7.04861 for glass bead sizes 2 (level 1), 4 (level 2), and 6 mm (level 3), respectively. A, C, and A·C stand for the variables time, bead/olive ratio, and their interaction.

According to the levels of glass bead sizes, the LAB releases are parallel planes in a three-dimension plot, but their overlapping makes visualisation difficult. Their general trends may be illustrated just by one of them, level 3 (6 mm) glass bead size and 300 rpm, as a function of the interaction time·bead/olive ratio (Fig. 1A). The LAB recovery increases as the time and bead/olive ratio increase, but the lowest bead/olive ratio is preferable for the longest time (Fig. 1 A). The 2D plot of the interaction time·bead/olive ratio at 400 rpm and 6 mm (level 3) glass bead size (Fig. 1B) shows that LAB recovery using a 0.16 bead/olive ratio increases with time, and it is above (but not significantly different than) those obtained with 0.50 glass bead ratio, which, in turn, did not change over time (Fig. 1B). The effect of time may be depicted by plotting (for example) LAB recovery as a function of time and glass bed size while setting the bead/olive ratio and agitation at 0.16 and 400 rpm, respectively (Fig. 1C). Time was critical because extraction for 15 min always led to the highest significant LAB counts (Fig. 1C). The level 3 (6 mm) glass bead size consistently led to the highest LAB recovery (Fig. 2A). Finally, the LAB recovery after 15 min was significantly higher than after 5 min when using a 0.16 bead/olive ratio; besides, the detachment during 5 min increased with the bead/olive ratio but slightly decreased when the time was 15 min. Thus, the LAB yield was similar for the 0.5 bead/olive ratio, regardless of time. That is, a higher bead/olive ratio had higher recovery when treating for 5 min but not for 15 min (Fig. 2B); on the contrary, using a 0.16 bead/olive ratio, one needs to extend the treatment for 15 min for the best results (Fig. 2B). A more convenient plot for the visualisation of the LAB recovery was by setting the glass bed size at the level of highest yield (6 mm) and agitation at that of the supposed most vigorous (but not significant) LAB removal (400 rpm), and plotting the contour lines of the RS as a function of bead/olive ratio and time (Fig. 2C). The conditions that led to the highest LAB release were located at the right-bottom of the figure (lowest bead/olive ratios and longest time), which agrees with all the previous comments.

Table 2
ANOVA (classical sum of squares. type II) regarding model fit to experimental data for lactic acid bacteria and yeasts. The adjusted response surface was expressed as $\log_{10}$ CFU/g.

Source	Sum of Squares	Df	Mean Square	F-Value	p-value Prob > F	Observations
Lactic acid bacteria						
Model	0.25	5	0.049	3.39	0.0247	Significant
A-time	0.1	1	0.1	6.99	0.0165	
C-Bead/olive ratio	0.018	1	0.018	1.26	0.2772	
D-Bead size	0.1	2	0.05	3.48	0.0528	
AC	0.043	1	0.043	2.96	0.1024	
Residual	0.26	18	0.014			
Lack of fit	0.21	16	0.013	0.56	0.798	Not significant
Pure error	0.047	2	0.024			
Total	0.51	23				
Yeast						
Model	0.22	6	0.037	2.89	0.0396	Significant
A-Time	0.055	1	0.055	4.27	0.0549	
B-Agitation	0.017	1	0.017	1.29	0.2712	
D-Bead size	0.048	2	0.024	1.84	0.1887	
BD	0.076	2	0.038	2.94	0.0803	
Residual	0.22	17	0.013			
Lack of fit	0.18	15	0.012	0.66	0.7463	Not significant
Pure error	0.037	2	0.18			
Total	0.44	23				

Notes: Some variables were introduced in the models to maintain the hierarchical characteristics. They were the following: lactic acid bacteria, C-glass-bead/ratio; yeasts, B-agitation, and D-glass bead size.

3.2.2. Yeast recovery

For yeasts recovery, the model suggested by the sequential sum of squares (Type II) was significant (p=0.0396), with only a 3.96% probability that an F value of 2.89 could be due to noise. The analysis retained the variable time and the interaction agitation·glass bead size (significant at $p \leq 0.10$) and the linear effects of agitation and glass bead size (included for maintaining the hierarchical conditions, Table 2, yeasts). The precision was 5.954 (higher than 4), which means an adequate response/noise ratio. Then, the model was adequate to navigate within the experimental region and consisted of an equation for each level of glass bead size (diameter). The functions, expressed in terms of actual factors, were:

$$\text{Yeast } (\log_{10} \text{CFU/g}) = I + 0.01159\ A + \beta \cdot B \quad \text{Eq 2}$$

Where I (+5.55286, +5.12696, and +5.19089) and β (+6.9195E-4, +7.3107E-4, and +8.3100E-4) stand for 2 (level 1), 4 (level 2), and 6 mm (level 3) of glass bead sizes, respectively. A and B stand for the variables time and agitation, respectively. Notice the low values of the agitation (B) linear coefficients (β) since its retention attends to the hierarchical requirement (because of its significant interaction). Then, in some way, this factor, significant for yeast detachment but not for LAB, conditions the level of this variable for the simultaneous detachment of LAB (non-affected) and yeasts.

A convenient interpretation of the results is obtained by studying the interaction time · glass bead size at the two extreme agitations (200 rpm, Fig. 3A, and 400 rpm, Fig. 3B) and an adequate 0.16 glass bead/olive ratio for LAB recovery (Fig. 4A–B). Notice that 15 min was always better than lower periods and that yeast recovery linearly increased with bead diameter at the highest agitation (400 rpm) (Fig. 3B), regardless of time. Besides, for 15 min and a 0.16 glass bead/olive ratio, the yeast recovery increases for beads levels 2 (4 mm) and 3 (6 mm) (Fig. 4A). Although agitation was irrelevant for LAB recovery, this trend confirms that the highest yeast detachment for level 3 of glass bead size (6 mm, also the best for LAB) increases as agitation is stronger. Then, setting the glass bead/olive ratio at 0.16 and bead size to level 3 (6 mm), the yeast detachment will depend on agitation and time (Fig. 4 B), with the best yeast recovery area on the top right corner (Fig. 4B). As a result, the simultaneous optimisation of LAB and yeast recovery is not simple but

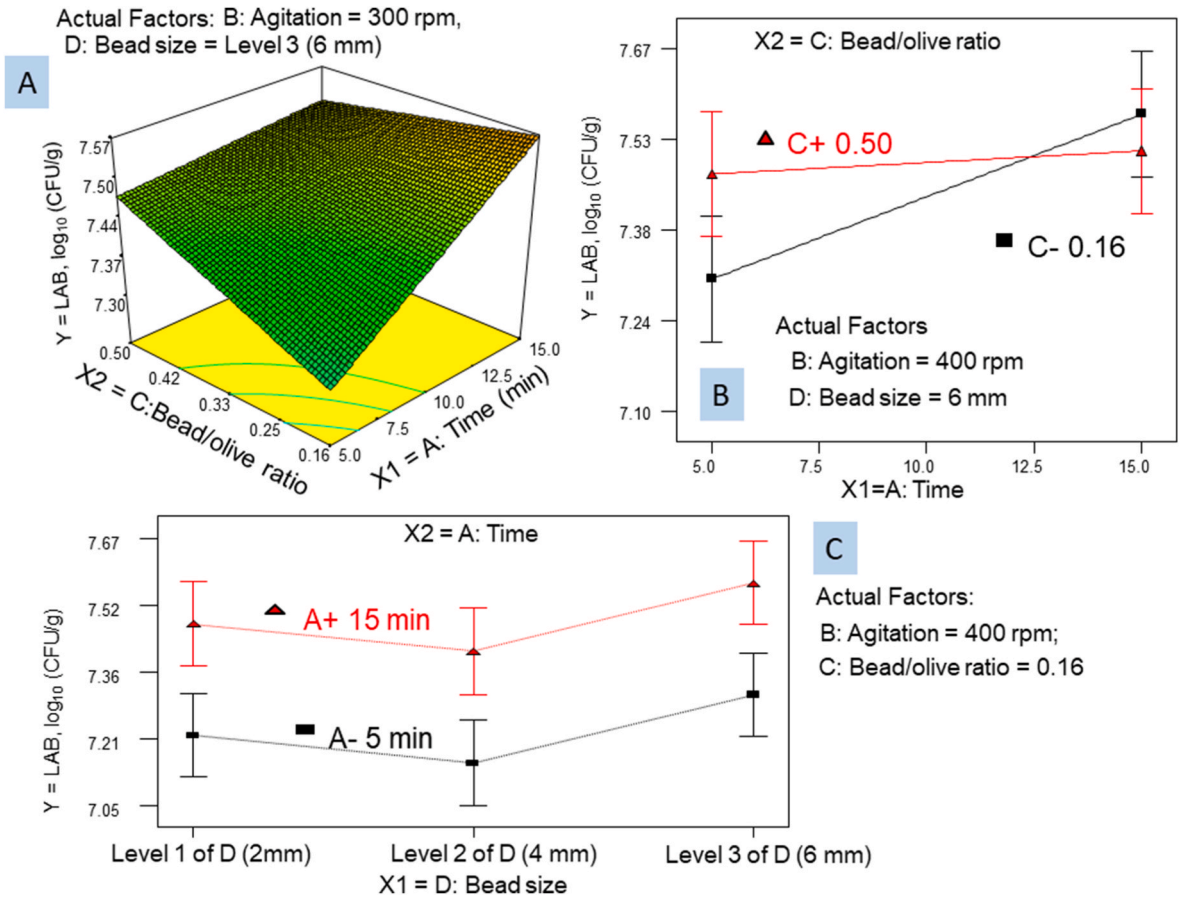

Fig. 1. D-optimal design to the LAB counts (log_{10} CFU/g) released by the glass bead methodology. A) Response surface. B) Interaction Bead/Olive ratio·Time; C) Interaction time·Bead size. The fixed values of the remaining actual factors are indicated in each plot.

requires the consideration of both LAB and yeast models.

3.2.3. *Simultaneous LAB and yeast recovery optimisation*

Ideally, the method should provide the highest recovery of both LAB and yeasts in a single extraction. The overall numerical optimisation proposed several solutions for 0.9125 desirabilities (Table 3), which always included time = 15 min, bead/olive ratio = 0.16, glass bead size 6 mm (level 3), and agitation ranging between 330 and 400 rpm (mainly because of agitation influence on yeast recovery). In this range of agitation, the predicted LAB recoveries were always 7.57 log_{10} CFU/g (3.72×10^7 CFU/g) since LAB release was independent of agitation, while the geometric mean (because of the log applied transformation) of yeast was 5.78 log_{10} CFU/g (6.026×10^5 CFU/g), with the variability only affecting the second decimal. In such agitation interval, it would be possible to get the best LAB recovery while slightly affecting yeast removal, provided that the other variables were set at the values suggested.

In addition, the values predicted by the models were compared with those obtained from the same olives by the stomacher (Table 1, last two rows; St 1 and St 2), whose geometric means were 7.66 and 4.46 log_{10} CFU/g (4.57×10^7 and 2.88×104^4 CFU/g, as counts) for LAB and yeasts, respectively. The levels found by the stomacher were higher and lower, respectively, than those predicted by the developed protocol, a situation that deserves attention. However, notice that, regardless of other considerations, the results should not be considered as evidence of the exhaustiveness of any of the methods.

Results obtained in this work are in line with previous applications of RSM in table olive research. López-López et al. (2016) used centroid mixture designs to study the effect of partial substitution of salt (NaCl) with other nutrient chloride salts. Results regarding the mineral content in flesh and the sensory characteristics of products allowed identifying the appropriate salts' mixtures to reduce sodium content while maintaining the typical features of the green Spanish style. Rodríguez-Gómez et al. (2012) used a simple centroid design to investigate the changes in LAB and yeasts during fermentation in different salt mixtures. Potassium chloride had a stimulating effect on the bacteria growth, while calcium chloride delayed the lactic acid production because of retarding the sugar released into the brine. Recently, Lahiri et al. (2021) used RSM to deduce the optimal conditions for the application of α-amylase for the *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm eradication. Dashti et al. (2016) used RSM to optimise *Salmonella typhi* biofilm formation in microtiter plates, finding that the methodology was a practical approach for biofilm assay optimisation. In this work, RSM was also appropriate to deduce the best conditions to release the microorganisms embedded in the table olive biofilm.

3.3. *Application of the developed glass bead protocol*

The glass bead protocol (15 min; bead/olive ratio, 0.16; glass bead size, 6 mm; and several rpm agitations within the suggested interval) and stomacher were also applied to olives from another similar but independent, Spanish-style process. The assays were performed with two olives, except for one 350 rpm duplicates tested with 4 fruits (350f). The data expressed as CFU/g and log_{10} CFU/g (Table 4) were studied by ANOVA, applying the Benjamini-Hochberg approach to account for the reduction in probability caused by multiple comparisons.

For the LAB results, the counts found by the stomacher were significantly higher than those from glass beads, regardless of the expression form. Besides, non-significant differences between most agitation levels were observed using just counts, except for 400 rpm, which was

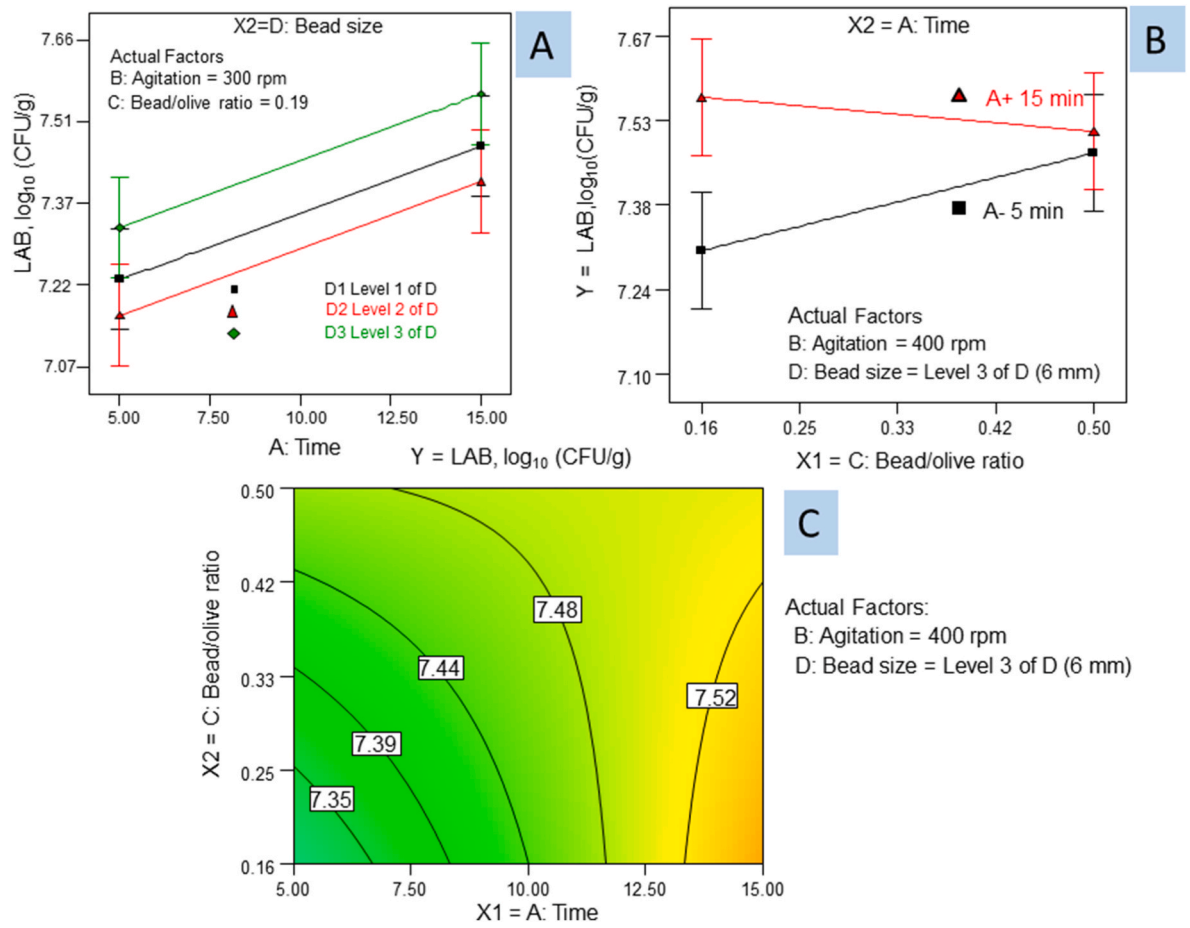

Fig. 2. D-optimal design to the LAB counts (log_{10} CFU/g) released by the glass bead methodology. A) Interaction Bead size·timer; B) Interaction Time·Bead/olive ratio; C) 2D contour lines of the interaction Bead/olive ratio·time. The fixed values of the remaining actual factors are indicated in each plot.

significantly lower only when data were expressed as log_{10} (CFU/g). The use of four olives led to similar statistical values as those from 2 fruits. On the contrary, no differences between the glass beads and stomacher were observed for yeasts, irrespective of the expression form. Then, when presenting olives as probiotics, CFU/g might be preferred. The LAB results in this independent assay were in line with those predicted and obtained with the stomacher in the design experiment.

The diverse trends observed between glass beads and stomacher protocols concerning LAB and yeast recovery suggest that their respective cell sizes play an important role. Possibly, the LABs, because of their smaller cellular volume, could adhere to the biofilm, the stomas' caverns, or even navigate inside the olive flesh through the intercellular spaces. Then, a variable fraction of the LAB population may colonise the stomas and the flesh while yeasts almost exclusively colonise the olive surface biofilm. The LAB in the depths of stomas or the olive flesh (mainly) might not be affected by the glass beads but counted using the stomacher. On the contrary, the yeasts (of bigger size than LAB) could be principally embedded in the biofilm adhered to the olive surface. The oscillations observed for yeasts by glass beads can be due to the disintegration degree of the olive biofilm. Research for a better understanding of the phenomenon is underway.

The hypothesised LAB and yeast distribution between biofilm and flesh in Spanish-style green olives are in line with previous studies on black olives. Scanning electron microscopy (SEM) has shown that LAB occupies the stomatal openings and colonises the intercellular spaces of substomal cells in mature olives (Grounta & Panagou, 2014; Nychas et al., 2002). In Spanish style, penetration in the stomas and the interior of the flesh is more difficult because of the more robust intercellular structure and the more rigid texture than mature olives. The distribution will depend on the olive characteristic and processing conditions.

3.4. *Metataxonomic analysis*

During stomacher homogenisation, the olive fruit breaks, releasing the microorganisms and disintegrating the fruit's plant cells, but such a process may be harmful when carrying out the metagenomic study of the olive biofilm. The analysis of amplicon data is complex if a great quantity of non-desired eukaryotic DNA, such as chloroplastic and mitochondrial commonly released from vegetable samples, are present since they provoke interferences with the bacterial or fungal data. The homology between bacterial 16S, chloroplast 16S, plant nuclear and mitochondrial 18S rRNA genes (18S), and arthropod 18S leads to challenges in choosing the appropriate primer pairs (Hanshew et al., 2013). For example, the universal 16S primers targeting hypervariable regions V1–V3 (27F, 338R, 519R), V3–V6 (534F, 926F, 1114R), and V6–V8 (926F, 1392R), used in some insect systems (Fagen et al., 2012; Jones et al., 2013), are homologous to chloroplast 16S (Lane, 1991; Rastogi et al., 2010). Some authors have focused their effort on designing primers intended to amplify bacterial 16S sequences while avoiding chloroplast 16S sequences (Beckers et al., 2016; Chelius & Triplett, 2001; Hanshew et al., 2013). However, designed primers have been sufficient for some plant systems (Rastogi et al., 2010; Sun et al., 2008) but not entirely effective in eliminating amplification of chloroplast 16S sequences in all samples (Bodenhausen et al., 2013; Shade et al., 2013). Chloroplasts are evolutionarily descendent from bacteria, so it is not surprising that the 16S genes were nearly homologous. Added to the current databases' low efficiency, DNA from chloroplastic and mitochondrial leads to habitually incorrect assignations or unassigned

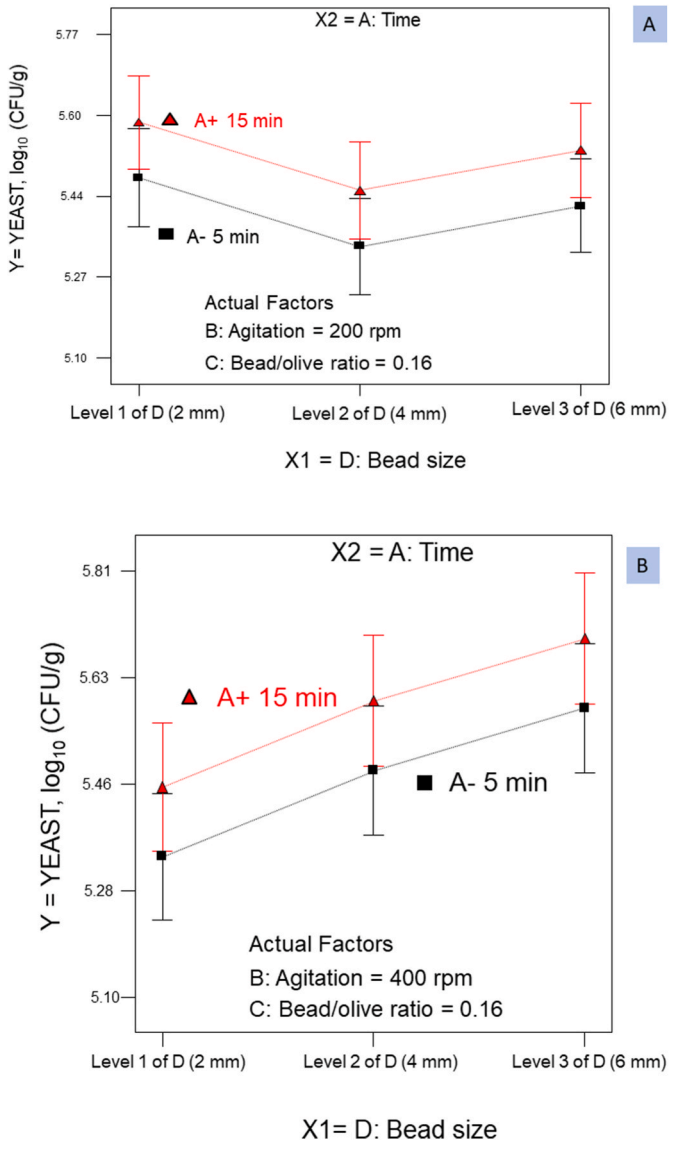

Fig. 3. D-optimal design to the yeast counts (log_{10} CFU/g) released by the glass bead methodology. Interaction Time·Bead size. A) Agitation, 200 rpm. B) Agitation, 400 rpm. The fixed values of the remaining actual factors are indicated in each plot.

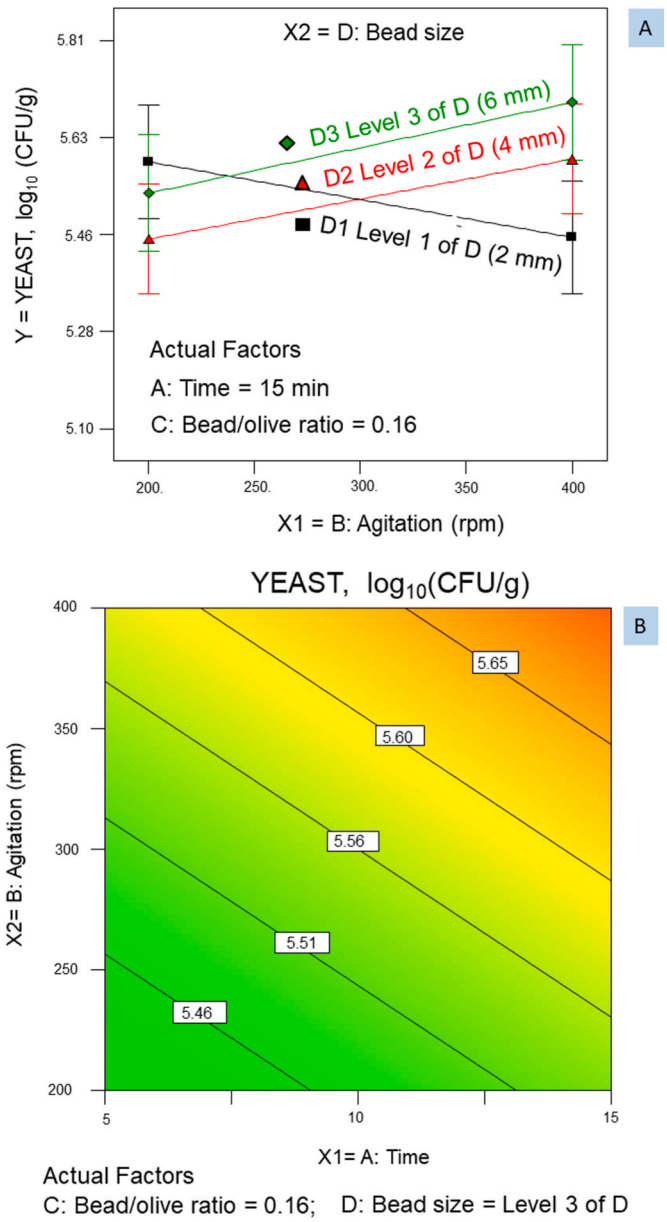

Fig. 4. D-optimal design to the yeast counts (log_{10} CFU/g) released by the glass bead methodology. A) Interaction Bead size·Agitatio; B) Contour lines of the interaction Agitation·Time. The fixed values of the remaining actual factors are indicated in each plot.

sequences. Thus, retrieving a precise taxonomical classification of the short metagenomic reads (100–250 bp) remains challenging (Breitwieser et al., 2019). They are still highly dependent on suitable non-destructive methods for the recovery of the microorganisms and good reference databases for a correct taxonomic assignment (Pedrós-Alió et al., 2018).

The study of the number of raw and filtered sequences for each filtering step obtained from samples processed with both glass beads and the stomacher methodologies after the metataxonomic analysis of the 16S and ITS regions (Table 5) showed that most of the sequences were retained in the first filtering step and mainly during the filtering of chimaeras (data not shown). For all steps assayed, the standard error obtained for the stomacher's data was markedly higher than for those extracted with glass beads samples (Table 5).

An average value of 126,0190 ± 9888 raw 16S sequences was generated from the samples processed with glass beads, of which a total of 40.44% were retained during the quality filtering steps. On the contrary, 120,313 ± 14,086 raw sequences were generated for the stomacher samples, being retained in 46.83% of them. No mitochondria were detected in any of the samples. Filtered 16S rRNA gene amplicons from glass beads samples were assigned to 12 different bacteria ASVs (including non-filtered chloroplasts), but on the contrary, only 4 ASVs were assigned in the stomacher samples. A total of 0.49% of glass beads sequences were assigned to chloroplasts, while in stomacher samples, they reached up to 41.2%. Non-assigned sequences (including chloroplast) were 42.77 and 0.51% for samples extracted with stomacher and glass beads, respectively (Table 5). In recent metataxonomic studies applied to samples from table olive processing, around 65% chloroplast and mitochondrial sequences from fruit cells have been obtained (Benítez-Cabello et al., 2019; Rodríguez-Gómez et al., 2017), although it could reach up to 90% (Medina et al., 2016). In other fermented foods, the sequences assigned to chloroplasts were around 43% (Mayo et al., 2014).

Regarding the bacterial taxonomy, 99.45% of sequences from glass bead samples were assigned to the genus *Lactiplantibacillus,* while the

Table 3
Numerical optimisation solutions. Factor combinations for the highest desirability (LAB and yeast simultaneous recovery, $\log_{10}$ CFU/g), using the glass beads procedure.

Solution number	Time (min)	Shaking intensity (rpm)	Glass bead/olive ratio	Glass bead size level	LAB $\log_{10}$ CFU/g	Yeast $\log_{10}$ CFU/g	Desirability
1	15	380	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
2	15	384	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
3	15	337	0.16	3	7.57 (0.06)	5.77 (0.05)	0.9125
4	15	323	0.16	3	7.57 (0.06)	5.76 (0.05)	0.9125
5	15	324	0.16	3	7.57 (0.06)	5.76 (0.05)	0.9125
6	15	344	0.16	3	7.57 (0.06)	5.78 (0.05)	0.9125
7	15	381	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
8	15	389	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
9	15	328	0.16	3	7.57 (0.06)	5.77 (0.05)	0.9125
10	15	395	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
11	15	392	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
12	15	353	0.16	3	7.57 (0.06)	5.78 (0.05)	0.9125
13	15	347	0.16	3	7.57 (0.06)	5.78 (0.05)	0.9125
14	15	348	0.16	3	7.57 (0.06)	5.78 (0.05)	0.9125
15	15	393	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125
16	15	400	0.16	3	7.57 (0.06)	5.77 (0.07)	0.9125
17	15	330	0.16	3	7.57 (0.06)	5.77 (0.05)	0.9125
18	15	357	0.16	3	7.57 (0.06)	5.78 (0.06)	0.9125

Note: Square error means in brackets.

Table 4
Comparison of the results obtained by applying the optimized glass bead protocol at three agitation levels and stomacher to olives from a new independent fermented batch.

Treatment	ANOVA on counts		ANOVA on $\log_{10}$
	LS mean CFU/g	Log_{10} (LS CFU/g) *	LS mean $\log_{10}$ CFU/g
LAB			
St	34987765 (3084898) [a]	7.544	7.532 (0.073) [a]
350 rpm	15193284 (2211352) [bc]	7.182	7.169 (0.052) [b]
300 rpm	12117780 (2211352) [bc]	7.083	7.080 (0.052) [b]
400 rpm	7893292 (2211352) [c]	6.897	6.885 (0.052) [c]
350f rpm	18558656 (3084898) [b]	7.269	7.268 (0.073) [b]
Yeast			
St	3842 (2704) [a]	3.585	3.569 (0.164) [a]
350 rpm	5916 (1912) [a]	3.772	3.742 (0.233) [a]
300 rpm	5592 (1912) [a]	3.748	3.578 (0.233) [a]
400 rpm	3726 (1912) [a]	3.571	3.498 (0.233) [a]
350f rpm	3312 (2704) [a]	3.520	3.468 (0.164) [a]

Notes: Treatment conditions were: 3 min at 300 rpm for the stomacher (St); 15 min, 0.16 bead/olive ratio, 6 mm (level 3) bead size, at 300, 350 and 400 rpm, for glass bead. Glass beads treatments were run in quadruplicate and using 2 olives, but stomacher (2 olives) and 350f rpm (4 olives) in duplicate. LS stands for the least square means estimated through the ANOVA analysis (standard error in brackets). Data in the same column followed by distinct super index letters stand for statistical differences ($p \leq 0.05$) according to LS means *post-hoc* comparison tests. The procedure Benjamini-Hochberg was used to consider the effect of multiple comparisons on probability. Log_{10} (LS CFU/g) is the $\log_{10}$ of the LS mean deduced for counts in the ANOVA.

value for this genus in stomacher samples was only 58.28%. This is a logical result because the inoculum added was a mix of three *Lactiplantibacillus pentosus* strains, together with *Lactiplantibacillus plantarum*, the most relevant LAB species responsible for lactic acid fermentation of table olives (Hurtado et al., 2012). The rest of the bacterial genera represented less than 0.1% of total sequences (Fig. 5).

Concerning the ITS region, samples from the glass bead methodology generated fewer raw sequences (80,423 ± 742) than those from stomacher (116,657 ± 10,308). During the filtering step, 37.11% of the sequences generated from the first samples were retained, while the proportion for the second one reached 57.41%. Filtered ITS amplicons from glass bead samples were assigned to 21 fungal ASVs. In contrast, sequences from stomacher samples were assigned to only 8 different

Table 5
Comparison of different parameters from the metagenomics analysis obtained by stomacher and glass beads biofilm detachment protocols. Average values (standard error in brackets) were estimated from duplicated samples performed under an independent biofilm detachment process.

	Stomacher	Glass beads
DNA amount (ng/uL)	6.91 (5.64) [a]	3.20 (0.48) [a]
Sequences/sample for 16S analysis.Input.	120313 (14086) [a]	126019 (9888) [a]
Sequences/sample for 16S analysis.Final.	68592 (8539) [a]	78738 (6454) [a]
Sequences/sample for ITS analysis.Input.	116657 (10308) [a]	80423 (742) [a]
Sequences/sample for ITS analysis.Final.	54741 (8674) [a]	52305 (760) [a]
Percentage of 16S sequences not assigned	42.77 (8.51) [a]	0.51 (0.11) [b]
Percentage of ITS sequences not assigned	63.59 (17.17) [a]	1.39 (0.88) [b]

Note: Distinct super index letters in the same row stand for statistical differences ($p \leq 0.05$) according to LS means *post-hoc* comparison test.

ASVs. Moreover, only 1.32% of sequences obtained from glass beads corresponded to unclassified reads, but in the case of stomacher samples, this number reached 61.46%. Both kinds of samples were practically dominated by the genera *Wickerhamomyces* (Fig. 5). *Wickerhamomyces anomalus* was one of the yeast species used as a starter culture at the 96.54% in samples extracted with glass beads but reached only 37.95% in samples treated beginning of fermentation. This yeast species is well adapted to the stressing fermentation conditions which govern Spanish-style green table olive fermentations (Arroyo López et al., 2008, 2012).

4. Conclusions

The use of RSM helped to develop a non-destructive protocol for the simultaneous recovery of LAB and yeasts from the olive biofilms, with the following operational conditions: time, 15 min; shaking, 350 rpm; glass bead/olive ratio, 0.16; and glass bead diameter, 6 mm for the simultaneous recovery of LAB and yeasts. The use of glass beads led to slightly/significantly lower LAB recovery than stomacher but statistically higher/similar of yeasts. It is hypothesised that stomacher includes microorganisms from both biofilm and olive flesh, while glass beads only release those embedded in the olive biofilm adhered to the fruit epidermis. Further studies to confirm that glass beads only recover those LAB and yeast in the olive biofilm are in progress. Moreover, another essential advantage of the new glass bead methodology is the practical absence of chloroplastic and mitochondrial DNA in the metagenomic analysis, which increases the number of assigned bacterial and fungal taxa.

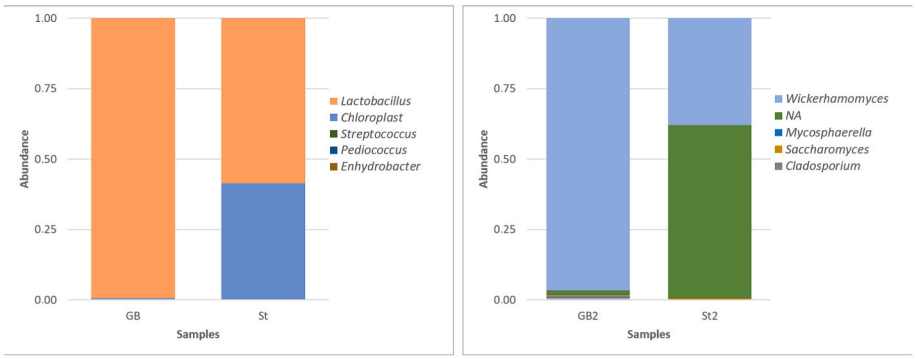

Fig. 5. Abundances of LAB and yeast genera were obtained after metataxonomic analysis using the glass bead (GB) and stomacher (St) detachment biofilm methods. Mean values were obtained from duplicated experiments.

CRediT authorship contribution statement

Elio López-García: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Antonio Benítez-Cabello:** Conceptualization, Data curation, and, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Virginia Martín-Arranz:** Methodology. **Antonio Garrido-Fernández:** Conceptualization, Data curation, and, Formal analysis, Writing – original draft, Writing – review & editing. **Rufino Jiménez-Díaz:** Funding acquisition, Project administration. **Francisco Noé Arroyo-López:** Conceptualization, Data curation, and, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This research was funded through the TOBE project (RTI2018-100883-B-I00, MCIU/AEI/FEDER, UE). ELG thanks the Spanish Ministry of Science and Innovation for his FPI contract (PRE2019-087812). ABC thanks to the Junta de Andalucía for his contract (PAIDI2020- 00162).

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