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The use of starter cultures in the table olive fermentation can modulate antiadhesive properties of brine exopolysaccharides against enterotoxigenic *Escherichia coli*.

Yanan Zhu[a], Gemma González-Ortiz[a], Antonio Benítez-Cabello[b], Beatriz Calero-Delgado[b], Rufino Jiménez-Díaz[b], Susana María Martín-Orúe[a,*]

[a]Animal Nutrition and Welfare Service, Animal and Food Science Department, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

[b]Department of Food Biotechnology, Instituto de la Grasa (Consejo Superior de Investigaciones Científicas), 41013 Seville, Spain.

*Corresponding author.

E-mail address: susana.martin@uab.cat (Susana María Martín-Orúe).

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Abstract

The present study aimed to evaluate different mates of *Candida boidinii* and *Lactobacillus pentosus* strains as starters in green table-olive fermentation. Changes in fermentation characteristics as well as changes in the functional properties of the microbial exopolysaccharides (EPS) produced during the process were registered. *In vitro* adhesion test demonstrated most EPS samples could specifically attach ETEC K88. *In vitro* studies with porcine intestinal cells showed improved blocking activity of the fimbria (blocking test) when the mutant strain *L. pentosus* 119-14MT was used alone as starter. All EPS samples showed the ability to block receptors in the cells (exclusion test) although without differences between starter treatments. In the displacement test, EPS samples failed to remove the pathogen once attached. According to these results, *L. pentosus* 119-14MT, a high EPS variant, seemed to be the most effective starter improving the anti-adhesive

properties of brine EPS, increasing its ability to block the ETEC K88 fimbria. These results illustrate that anti-adhesive properties of the EPSs produced during the traditional fermentation of olives could be modulated by the use of defined starters. This open the door to new fermentation processes aimed to produce green table olive as functional food to prevent ETEC diarrhea.

1. Introduction

In Mediterranean regions, green table-olives, which are also called Spanish or Sevillian style, is a particularly important industry. During the year 2017-2018, the production of table olives in the world was estimated at 2.95 million tons, and Spain produced about 521,500 tons (17.68 %).[1]

Green table-olives is a fermented food. For their manufacturing, oleurpein in the olive fruit is firstly eliminated by a diluted lye solution (sodium hydroxide), and after a water wash, green olives are covered with brine in suitable containers, and then the fermentation is carried out spontaneously for several months.[2,3] During the fermentation, hundreds of bacteria and yeast have been identified by molecular techniques to participate in the process.[4] Lactic acid bacteria (LAB) have been considered the main bacteria responsible for the fermentation by transforming sugars into lactic acid.[5] Yeast also plays important roles during the fermentation, as they can provide LAB with vitamins, amino acids and purines, and improve LAB growth.[6] Actually, many of these microbial species have been described to grow on polymicrobial biofilms covering biotic (skin of the olives) and abiotic (fermenter walls) surfaces.[7,8]

The predominant LAB-forming biofilm on olive surfaces is *L. pentosus*, although many other yeast species have also been described to coexist in the same biofilms.[9] In particular, works of León-Romero et al.[10] described co-aggregative interaction between different strains of *L. pentosus* and *Candida boidinii* isolated from natural green-olive fermentations when they were co-cultured together *in vitro*.

To build biofilms, microbial communities surround themselves with a matrix of exopolysaccharide material (EPSs) and extracellular DNA. This matrix allows bacteria to share nutrients, communicate ant protect themselves from harmful factors in the

environment.[11] However, together with these; a biofilm matrix could also have other still unknown biological functions. Previous works of our group firstly described how EPS isolated from olive brines had abilities to interfere with the adhesion of enterotoxigenic *Escherichia coli* (ETEC) K88 to the porcine intestinal mucus and intestinal porcine epithelial cells (IPEC-J2).[12,13] In subsequent research, the activity was compared of EPSs isolated from up to 5 different industrial olive fermenters, confirming their biological functions against ETEC K88, but also showing variability in the intensity of the anti-adhesive abilities, depending on the fermentation batch.[14] The variability of results suggests that naturally expected variations in the fermentative communities and environmental conditions in the fermenters could lead to compositional changes in the biofilm matrix and EPS functionality. Actually, previous research by León-Romero et al.[10] corroborated *in vitro* that the biofilm formation was a complex mechanism, probably affected by changes in environmental conditions that occur in the natural ecosystem provided by the olive fermentations, and that biofilm formation could be favored by a specific mate of yeast and *L. pentosus*strains. Until now, there is no research regarding the possible effects of using particular olive fermentation starters on the anti-adhesive properties of the EPSs produced by biofilms on olive surfaces. It is therefore hypothesized that, by using defined starter cultures of *L. pentosus* and *C. boidinii*, it could be possible to improve the bio-functions of the microbial EPSs isolated from green-olive brines.

In this study, four preselected *L. pentosus* strains (with different capacities to produce biofilms) were combined or not with *C. boidinii* TOMC-Y13, giving ten possible starters (2 × 5) that were used in 20 pilot fermenters. EPSs isolated from olive brines after 60 days of fermentation were evaluated in different miniaturized *in vitro* models for their ability to attach ETEC K88 and to interfere in its adhesion to IPEC-J2 cells.

2. Materials and Methods

2.1 Olive Fermentation

Manzanilla-variety fruits (4.05 g ± 0.49 g size) were obtained during the 2015/2016 season at the green ripening stage from JOLCA, S.A. (Huévar del Aljarafe, Seville, Spain), and transported to the laboratory, where they were de-bittered according to the Spanish style

(lye treatment with 2.3% NaOH). After washing (12h) to remove excess alkali, the fruits were brined in polyethylene fermenters with a capacity for 5.2 kg of fruits and 3.4 L of brine (10% NaCl). After 2 days to reach equilibrium, CO_2 was bubbled into fermenters to adjust the initial pH to below 6.0. Then, 20 pilot fermenters (2 per treatment) were subjected to 10 different treatments according to a 5 × 2 factorial design (4 *L. pentosus* strains starters + blank control) x (*C. boidinii* TOMC-Y13 + blank control) = 5 x 2 = 10). According to this design, two fermenters were not inoculated and were left to ferment spontaneously, and the other 18 received one of the 9 different microbial starters (2fermenters/starter). Initial starter doses per fermenter were adjusted to approximately 7 log10 CFU mL^{-1} (final concentration) for each of the following *L. pentosus* strains: 119WT, 119-14MT, 13B4WT, 13B4-13MT and to 5 log10 CFU mL^{-1} for the *C. boidinii*TOMC-Y13 strain (final concentration). The 119WT and 13B4WT strains of *L. pentosus* were previously isolated from diverse table-olive processing brines based on their technological and probiotic potential[15]. Non-coaggregative spontaneous mutants 119-14MT and 13B4-13MT were obtained as previously described[16]. The *C. boidinii* TOMC-Y13 strain was selected based on its technological and inter-species co-aggregative properties.[15] All of these microbial strains belong to the Table Olive Microorganisms Collection (TOMC) of the Instituto de la Grasa (CSIC; Seville, Spain).

2.2 Monitoring of the Fermentation

Physiochemical characters of titratable acidity (TA) (expressed as lactic acid, g/100 mL), combined acidity (CA) (expressed as undissociated organic salts, Eq/L) and pH were tested at different periods (0 days, 30 days, 60 days) according to the methods described by Garrido Fernandez et al.[17] The yeast and LAB populations adhered to the olive surface, or in the fermentation brines, were also tested, as in the description in the study by Benítez-Cabello et al.[18]

Briefly, microbial populations adhered to fruits were isolated by homogenizing clean and previously washed olives for 1 min at 300 rpm in a stomacher model Seward 400 (Seward Medical, Ltd.; West Sussex, England). Suspensions of the samples were then plated onto solid selective culture media. Enterobacteriaceae were counted on Violet Red Bile Glucose (VRBG) agar (Merck; Darmstadt, Germany), lactobacilli were spread onto de Man Rogosa

and Sharpe (MRS) agar (Oxoid; Basingstoke, Hampshire, England) supplemented with 0.02% (wt/vol) sodium azide (Sigma; St. Louis, MO, USA), and yeasts were grown on yeast-malt-peptone-glucose medium (YM) agar (Difco, Becton and Dickinson Company; Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate (0.005%, wt/vol) as selective agents. Counts were expressed as log10 CFU/g.

2.3 EPSs Isolation

The extraction of EPSs from olive-fermentation brines obtained after 60 days of incubation was carried out using the method described by Sánchez et al.[19] with some modifications. Briefly, brines were centrifuged at 10,000 × g at 4℃ for 30 min to get rid of suspended solids and bacteria. Then, 10% w/v trichloracetic acid (TCA) was added and stirred for 30 min at RT followed by centrifugation to remove the pellet. Two volumes of 96% cold ethanol (4℃) were used to precipitate the EPSs at 4℃ overnight. After this, the precipitated EPSs were recovered by centrifugation, re-suspended in ultrapure water and dialyzed in dialysis tubes of a 10kDa-14kDa molecular-mass cutoff (Medicell Membrane Ltd.; UK) for 48h at 4℃. Dialysates were subsequently lyophilized and stored at RT until use.

2.4 Miniaturized *in vitro* models

2.4.1 Preparation of EPS solutions for *in vitro* studies

Each lyophilized EPS sample was suspended in PBS at a solid-to-liquid ratio of 1:10 (w/v). These suspensions were vortexed and sonicated (J. P. Selecta; Spain) alternately three times for 30s each and then centrifuged at 460 × g for 5min (Mikro 220R, Hettich Zentrifugen). All supernatants were stored at -20℃ until used. Casein glycomacropeptide (CGMP) (Arla Foods, S. A.) was also included as a control in the different tests. Solutions of CGMP to be used in the tests were obtained following the same procedure.

2.4.2 *Escherichia coli* strains

In this research, two different strains of *E. coli* were used. ETEC K88 was generously provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo), which was isolated from a colibacillosis outbreak in Spain,[20] with

142 serotype (O149:K91;H10(K-88)/LT-I/STb). The other strain was a non-fimbriated *E. coli*
143 (NF-*E. coli*) (F4-, F6-, F18-, LT1-, ST2+, Stx2e-) that was kindly donated by the
144 Departament de Sanitat i d'Anatomia Animals of the Universitat Autonoma de Barcelona,
145 and was isolated from the feces of a post-weaning piglet. The ETEC K88 strain was grown
146 in unshaken Luria broth (LB) at 37ºC for 24h, and the non-fimbriated strain was cultured in
147 shaking LB. Finally, cultured bacteria were centrifuged (1,700 × g, 10 min., 20ºC) and the
148 cell pellet was re-suspended in PBS buffer and adjusted to an optical density (OD) of 1 at
149 650 nm (OD_{650nm}). For the adhesion test, adjusted suspensions were used directly (OD_{650nm}
150 = 1); for the *in vitro* test with IPEC-J2 cells, a dilution of 1/100 times of this suspension
151 was performed to reach a final concentration of 6.5-7 log CFU/ml in order to optimize the
152 bacteria/cells ratio.

153 **2.4.3 Cell-culture growth**

154 The IEPC-J2 cells (epithelial cells isolated from the jejunum of the neonatal piglet), kindly
155 donated by Dr. Antony Blikslager, from the College of Veterinary Medicine (North
156 Carolina State University), were cultured in Dulbecco´s Modified Eagle Medium
157 (DMEM)/Ham´s F-12 (GIBCO®, Ref.no.:31331-028, Life Technologies; Spain), with
158 insulin, transferrin, selenium and ethanolamine added as ITS solution (GIBCO®,
159 Ref.no.:41400-045, Life Technologies; Spain). Cells were maintained in an incubator at
160 37ºC with an atmosphere of 5% CO_2. Cells were used between passages 95 and 105 and
161 were routinely tested to be free of mycoplasma contamination. For the *in vitro* tests
162 (competition, exclusion and displacement tests described below), 2×10^4 cells were seeded
163 into 96-well flat-bottom plates (Nunclon Delta Surface, Ref.no.: 167008, Thermo
164 Scientific; Denmark) in a 200-µl volume. After 24h culture in an atmosphere of 5% CO_2 at
165 37ºC, cell confluence was confirmed under the microscope, and then cells were washed
166 once with PBS. Two-hundred microliters of CO_2-independent medium (GIBCO®, Ref.no.:
167 18045-054, Life Technologies; Spain) were added into each well, and cells were left in the
168 incubator (37ºC for 24h without CO_2) until use.

169 **2.4.4 Adhesion test (AT)**

The Adhesion test was implemented as described previously.[13] Briefly, 300 µl of the PBS soluble extracts (1:100 w/v) obtained from the different EPSs and CGMP (described above) were incubated in 96-well, high-binding polystyrene microtitration plates (Microlon F plate 655 092; Greiner Bio-One BV) at 4℃ overnight. After removing nonbinding material by a sterile PBS wash, wells were incubated with 1% bovine serum albumin and 0.5% sodium azide in PBS (w/v) at 4℃ for 1h to block non-specific adhesion sites. Following two washes with sterile PBS again, 300 µl of the bacteria suspensions (ETEC K88 or NF-*E. coli*) were incubated for 30 min at room temperature. Wells were washed three times with sterile PBS to remove the non-attached bacteria. Three-hundred milliliters of sterile Luria broth were added, and the sigmoidal growth of bacteria was measured in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37℃ for 12h at $OD_{650nm,}$ 10-minute intervals.[21] All readings were taken in two independent assays and in triplicate per trial.

2.4.5 Miniaturized Assays with IPEC-J2 cells

Miniaturized assays were performed as in the previous descriptions by González-Ortiz et al.[12] and Salcedo et al.[22] A dilution of 1/100 times of suspension (OD = 1) of *E. coli* was performed in order to optimize the bacteria/cells ratio.

Competition test (CT)

Solubilized EPS samples (1:100 w/v) were gently mixed with an equal volume of each *E. coli* strain suspension. These mixtures were immediately added to confluent monolayers of IPEC-J2 in 200-µl volume and were incubated with the IPEC-J2 cells at 37℃ for 30 min to allow non-blocked bacteria to adhere to cells. Wells were then washed once by gently pipetting with sterile PBS to remove the non-adhered bacteria, but without disturbing the cell monolayer.

Exclusion test (ET)

One-hundred microliters of solubilized EPS (1:100 w/v) were gently mixed with an equal volume of PBS. After this dilution, mixtures were immediately added to confluent monolayers of IPEC-J2 in 200-µl volume. IPEC-J2 cells were incubated with mixtures at 37℃ for 30 min to allow EPS to adhere to cells. Wells were gently washed twice with

sterile PBS to remove the non-adhered EPS, but without disturbing the cell monolayer. One-hundred microliters of *E. coli* culture were then mixed with 100 µl PBS, and IPEC-J2 cells were co-incubated with this mixture again at 37℃ for 30 min. Wells were washed twice by gently pipetting with sterile PBS to remove the non-adhered bacteria, but without disturbing the cell monolayer.

Displacement test (DT)

One-hundred microliters of *E. coli* culture were mixed with 100 µl PBS, and then were co-incubated with IPEC-J2 cells at 37℃ for 30 min. After washing twice with PBS solutions, IPEC-J2 cells were incubated with 200 µl EPS (0.5%) at 37℃ for 30 min. Wells were then washed twice as previously described.

The following steps were the same for the previous three tests. Two-hundred microliters of CO_2-independent medium were added to allow for the growth of the adhered bacteria and to keep cells alive. Plates were covered by a film (VWR, Cat No.6094-064) and monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37℃ for 12h at $OD_{650nm,}$ 10-minute intervals. All readings were taken in two independent assays and in triplicate per trial.

2.5 Analysis of OD data

The OD_{650nm} data from the tests were processed by non-linear regression analysis using the non-linear P-NLIN procedure (Gauss-Newton method) through SAS 9.2 (SAS Inc.; Cary, NC, USA) following the equations described by Becker and Galletti.[23] From the time at which the bacterial growth reached an $OD_{650nm}$of 0.05 the $t_{OD=0.05}$ value (in hours) was defined.

The final $t_{OD=0.05}$ values were translated into colony forming units (CFU) by correlations previously defined between $t_{OD=0.05}$ values and initial number of bacteria seeded in microplate wells. Cultivated ETEC K88 and NF-*E. coli* strains were serially diluted in LB medium and the CFU/ml determined by plate counting. At the same time, 300 µl per well of each dilution and bacteria were added into microtitration plates in three replicates. The plates were monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices

Corporation) at 37℃ for 18h, as previously described. Fitted equations were: y = -1.682x +13.916 (R^2 = 0.989) for ETEC K88 and y = -1.084x+9.364 (R^2 = 0.975) for NF-*E.coli*, where "y" corresponds to $t_{OD=0.05}$and "x" to the log of CFU per well.

Data from *in vitro* tests were also expressed as the **Δ** *t* observed between the $t_{OD=0.05}$values registered for each EPS sample and its PBS control included in a same assay (**Δ** *t* = $t_{OD=0.05}$(h) of EPS sample - $t_{OD=0.05}$(h) of PBS control). In AT, a lower negative **Δ** *t* value reflects the ability of EPS to attach more bacteria. In the other three tests with IPEC-J2 cells, a higher positive **Δ** *t* value means higher anti-adhesive effects with fewer attached bacteria to the cells.

2.6 Statistical Analyses

Significant differences between treatments were determined by a linear model with two-way analysis of variance (ANOVA), with the R v.3.3 free software. In term of results about **Δ** *t*, replication assay was included as a blocking factor. Values are presented as means ± SD. Differences between means were tested by the Tukey-Kramer adjustment for multiple comparisons.

3. Results

3.1 Physicochemical and microbiological changes in the fermentation

Table 1 shows changes in titratable acidity (TA), combined acidity (CA), and pH from the fermenters at Days 0, 30 and 60. Titratable acidity and CA clearly increased after the first 30 days of incubation but only slightly afterwards. After 60 days, TA decreased when *C. boidinii* was included in the starters (P = 0.07), but no significant changes were found related to the different *L. pentosus* strains. Combined acidity only showed significant differences related to the treatments at Day 0, when we compared fermenters that included or not *C. boidinii* as starters.

In accordance with changes of TA and CA, pH values decreased, the main decrease being observed between Day 0 and Day 30 (from 5.21 ± 0.13 to 4.33 ± 0.08). Over the last 30 days of fermentation, pH values only changed slightly, from 4.33 ± 0.08 to 4.26 ± 0.08. Regarding the effect of the starters, a trend was found for an increased pH at Day 60 when

C. boidinii was included (P = 0.06). There were also differences discovered related to the different *L. pentosus* strains, the highest value corresponding to mutant type 119-14MT, and the lowest to mutant type 13B4-13MT (P = 0.06). However, in any case, the magnitude of the differences was quite small.

Table 2 shows microbiological changes registered in the fermenters expressed as plate counts of lactobacilli and total yeast (log CFU/ml), both in the liquid media and in the olive surfaces. Total numbers of yeast did not show a consistent pattern along the time of incubation, and they increased or decreased depending on the treatment. In any case, counts of total yeast were not high and values ranged between 1.3 and 4.2 log CFU/ml for liquid brines and < 1 and 3.5 log CFU/ml for olive surfaces. Regarding lactobacilli, they clearly increased in the olive surfaces after 30 days of incubation and decreased afterwards (5.4 ± 0.80, 8.1 ± 0.60 and 6.2 ± 0.27 log CFU/mL for Days 0, 30 and 60, respectively). In the brine, lactobacilli showed a trend to decrease over time (8.7 ± 1.64, 7.7 ± 0.38 and 6.4 ± 0.40 log CFU/mL for Days 0, 30 and 60, respectively).

Regarding the effects of the starters on microbial communities of the fermenters, significant changes were registered in the number of yeast attached to the olive surfaces after 60 days of incubation related to the starter treatments (P interaction = 0.03). In general terms, the inclusion of *C. boidinii* TMOC-Y13 promoted an increase of more than 1 log unit in the number of yeast except when combined with the *L. pentosus* 13B4 13MT strain. In this case, it was not possible to quantify yeasts on olive surfaces (minimum detection level: 10 CFU/ml), suggesting that strain 13B4-13MT had prevented the colonization of the olives by the *Candida* strain or other yeast strains. On the other hand, when *C. boidinii* was not introduced as starter, yeasts were hardly countable on olive surfaces (levels < 10 CFU/ml), except if mutant strains of *L. pentosus* were used alone. In this case, countable yeasts could be registered on the olive surfaces (2.4 ± 0.29 CFU for 119 14MT and 0.9 ± 1.29 CFU for 13B4 13MT). Other changes in microbial counts were registered with the different starter treatments. A statistical trend was found in the numbers of lactobacilli on olive surfaces at Days 30 (P = 0.07) and 60 (P = 0.06) due to the different *L. pentosus* strains used. Nevertheless, no change was detected, as related to the inclusion of *C. boidinii* as starter and no interaction between lactobacilli and *Candida* strains.

3.2 Impact on the ability of EPS to attach ETEC K88 and to interfere with its adhesion to IPEC-J2 cells

Fig. 1 shows the ability of EPS isolated from green-olive brines to attach ETEC K88 and to interfere with its adhesion to IPEC-J2 cells. Data are expressed as log CFU/well for the different miniaturized *in vitro* models, and CGMP and PBS were included as positive and negative controls, respectively. Results clearly show how EPSs have ability to specifically attach ETEC K88 in a similar degree as does CGMP. Regarding the impairment of adhesion of ETEC K88 to IPEC-J2 cells, olive brine EPS reduces numerically the number of adhered bacteria, as compared to PBS in the CT, although differences did not reach statistical significance (P = 0.26). In the ET, EPS obtained from fermenters including starters significantly reduced the number of adhered ETE K88 cells (P = 0.02). However, we were not able to detect significant changes in the DT test. As expected, results obtained with NF-*E. coli* revealed no significant difference between EPS treatments.

Regarding the effect of the different starters on the functionality of the isolated EPS, Table 3 shows the results obtained from the different miniaturized models expressed as $\Delta t_{OD=0.05}$values (h), comparing each EPS sample with its PBS control within each assay. Results from the AT showed decreases in $t_{OD=0.05}$ of around one hour, which would confirm the ability of EPS to attach ETEC K88. Nevertheless, we could not see differences between the starter treatments in this ability ($P < 0.05$). Regarding the studies with IPEC-J2 cells, in the CT (ability to block fimbria), increases were found in $t_{OD=0.05}$that ranged from 0.03 ± 0.40 to 0.57 ± 0.08 h (P interaction = 0.05; See Fig. 2), showing that the blocking activity depended largely on the kind of starter used. Fig. 2 shows the highest blocking activity for strain 119-14MT (P = 0.04), particularly when not combined with *C. boidinii* TOMC-Y13. Wild-type strain 119WT also showed blocking activity, but this ability almost disappeared when combined with *C. boidinii*. This interaction was not observed for strain 13B4, which showed lower increments in the $t_{OD=0.05}$values. These results would demonstrate that the use of *L. pentosus* 119 as starter, and particularly the mutant variant, improve the blocking activity of the EPS, but its combination with *C. boidinii* impairs this activity. These results confirm our initial hypothesis, stating that the use of defined starters could improve the antiadhesive properties of the brine-isolated EPSs.

The ET results (ability to block receptors in the cells) showed reductions in $\Delta t_{OD=0.05}$ of around half an hour without differences between starter treatments, and the DT failed to demonstrate the ability of any EPS samples to remove the pathogen once attached, regardless of the starter treatment.

4. Discussion

Carbohydrate related structures of receptors on cell surface are the main biofunctional sites for ETEC K88 adhesin recognition.[24] Several researches have verified that natural ingredients or extracts containing carbohydrates acts as fimbriae or receptor analogues in prevention of bacterial infections.[25] In our previous studies, EPS isolated from green olive brines could reduce adhesion of ETEC K88 to porcine intestinal mucus and IPEC-J2 cells.[12,13] Further research confirmed the anti-adhesive abilities of EPSs from 5 different olive fermenters with variable intensity in the anti-adhesive activity depending on the fermentation batch.[14] Those results suggested that differences in the microbial consortia involved in the fermentation of green olives could lead to changes in the composition and structure of the produced EPS and therefore in their activity.

Considering these previous results, in this study we wanted to test how different combinations of *L. pentosus* strains, differing in their ability to produce EPS, and the yeast *C. boidinii*, introduced as starters in the fermentation of olives, could induce changes in the biofilm formation and characteristics and properties of EPS produced.

4.1 Physicochemical and microbiological characterization of fermentation

Titratable acidity (TA) and combined acidity (CA) can be used to calculate combined, free, and corrected total acidity.[26] For all treatments, CA and TA increased significantly in the first 30 days and slowly in the next 30 days in a similar way. Different olive starters did not influence contents of TA or CA in the 30^{th} or 60^{th} day.

In accordance with changes of TA and CA, pH values in brines significantly declines in the first 30 days, and in the following 30 days there was no huge change. In the studies conducted by Blana et al.[27] and Dalla Rosa et al.[28] authors also found similar phenomena. Moreover, different *L. pentosus* strains or *C. boidinii* showed trends to influence the pH

343 values at the 60th day (Both $P = 0.06$). Previous research also verified how different olive
344 starters could induce changes in pH values during the fermentation process, especially in
345 the terminal periods.[27,28] The reason was probably related to different metabolism of
346 bacteria. For example an increase in the growth of LAB bacteria, responsible to translated
347 sugars into lactic acid, could be associated to decreases in the pH.[5] Actually in our study the
348 inclusion of *C. boidinii* in the fermenters was associated to a decrease in the numbers of
349 LAB associated to the green olive surfaces ($P = 0.01$) and to an increase of pH at day 60.

350 Microorganisms do not exist separately but rather coexist in more or less complex
351 ecosystems. In this regard, it is known that on olive surfaces, *Lactobacillus pentosus* and
352 yeast species coexisted in biofilms,[9] and that yeast can improve LAB growth by providing
353 vitamins, amino acids and purines.[6] Here the results can be remembered of León-Romero et
354 al.,[10] who verified *in vitro* the complexity of interactions between different strains of *L.*
355 *pentosus* and *C. boidinii* in the formation of biofilms. Results from this study would
356 suggest that *L. pentosus* mutant strains could selectively favor the building of biofilms
357 together with yeast naturally present in the olives strains. In the particular case of the 119
358 14MT and *C. boidinii* combination, the lack of countable yeasts on olive surfaces would
359 suggest the competitive displacement of indigenous olive yeasts after 60 days of
360 fermentation, and the inability of both strains to build biofilms together. Therefore, we
361 could hypothesize that in our study the introduction of *C. boidinii* TOMC-Y13 in the
362 starters would have led to specific changes in the fermentation process that competitively
363 had favored the growth of yeast on olive surfaces. Despite this, when this starter was
364 combined with *L. pentous* strain 13B4 13MT, the effect was the opposite, with an exclusion
365 of *C. boidinii* from olive surface biofilms.

366 **4.2 Influence of fermentation starters on EPS interfering with ETEC K88 adhesion**

367 Confirming previous observed results,[14] EPS obtained from the starter-controlled
368 fermenters significantly increased the number of attached bacteria in the AT and also
369 reduced the number of ETEC K88 attached to IPEC-J2 cells in the ET (Fig 1.). These
370 results confirm the ability of these complex carbohydrates to interfere in the adhesin-
371 receptor recognition. However we were not able to detect, in this case, significant changes

372 in the CT or DT. Differences in the nature of EPS between studies and also in the scale of
373 the fermenters (pilot or industrial) could be behind these differences.

374 Interestingly we could find some differences in the blocking activity of the isolated EPS
375 related to the starters used, confirming the initial hypothesis. Different *L. pentosus* starters
376 influenced the ability of the EPSs to block fimbria in the CT being impaired when 119WT
377 and 119-14MT strains were used combining with *C. boidinii* TOMC-Y13. From the tested
378 combinations of starters the best results were found for 119-14MT when it was not
379 combined with *C. boidinii* TOMC-Y13 and the lowest activity for 119-14WT when
380 combined with the yeast. These results confirm the potential of using defined starters to
381 improve the antiadhesive properties of olive brines EPS. Using as starter, the mutant type
382 strain of *L. pentosus* as 119-14MT, able to produce a higher amount of EPS, appears
383 therefore as potential strategy to control olive fermentation for the production of EPS with
384 higher ant adhesive properties.

385 Multiple factors could be behind the impairment of bacteria adhesion by EPSs.
386 Physicochemical and/or structural characteristics of microbial EPSs have been considered
387 as vital factors in the probiotic and enteropathogenic adhesion to human intestinal mucus.[29]
388 These characteristics have been demonstrated to be easily influenced by culture conditions,
389 which can determine the type and concentration of EPS produced.[19,30] Even the same
390 bacteria strain has been demonstrated to be capable of synthesizing different EPSs under
391 similar conditions.[31] As previously shown, microbiota participating in green-olive
392 fermentation do not exist separately, but rather in complex ecosystems. Yeast species and
393 *Lactobacillus pentosus* during olive fermentation coexisted in the same biofilms on olive
394 surfaces.[9] León-Romero et al.[10] further explained that *in vitro* co-culture of different mates
395 of *L. pentosus* and *C. boidinii* strains isolated from natural green-olive fermentations
396 formed biomass and biofilms to a different extent. Based on these results, it was easy to
397 understand in our study that changes promoted in the fermentation process by the use of
398 particular starters would have affected the kind of EPS produced, and therefore their
399 functional properties. Specifically, results obtained in the CT evaluating the ability of EPS
400 samples to competitively bond with ETEC K88 fimbriae, suggest that the mechanism
401 involved in this change of activity is an increase in the recognition of bacterial fimbria by

isolated EPS when strain 119-14MT is used. The reduction of activity observed for this strain, when used in combination with *C. boidinii* TOMC-Y13, would suggest that this consortium could modify the way by which *L. pentosus* colonizes the olives and forms biofilms. Changes in the attachment of yeast and/or LAB to olive surfaces could have directly modulated the amount and characteristics of the EPS produced. Actually, the changes observed in the number of yeast attached to the olive surfaces after 60 days of fermentation would support this hypothesis.

5. Conclusions

By using different strains of *L. pentosus* and *C. boidinii* as starters in the production of table green olives, it is possible to modulate the fermentation process and improve the anti-adhesive properties of the microbial EPS isolated from the brines. The mutant strain *L. pentosus* 119-14MT, high EPS-producer, spontaneous variant of *L. pentosus* 119, appears as the most effective starter to improve the antiadhesive properties of isolated EPS, increasing its ability to block the ETEC K88 fimbria. This improvement is lost when this strain is combined with *C. boidinii* TOMC-Y13.

Conflicts of interest

There are no conflicts of interest to declare.

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528

Table 1 Effects of microbial starters on the physicochemical characteristics of the fermentation of table green-olives. Variables included pH, titratable acidity (TA) and combined acidity (CA) of the brine at Days 0, 30 and 60 of fermentation.

		Main effects										
		C. boidinii		*L. pentosus* strains						*P*-Value		
	day	+	-	119 WT	119-14MT	13B4 WT	13B4-13MT	N	RSE	C	L	C × L
pH	0	5.21	5.21	5.15	5.29	5.22	5.16	5.23	0.111	0.89	0.44	0.08
	30	4.31	4.36	4.34	4.41	4.33	4.28	4.31	0.073	0.13	0.24	0.51
	60	4.29	4.23	4.29	4.31	4.24	4.18	4.29	0.058	0.06	0.06	0.09
TA (g/100 ml)	0	0.14	0.14	0.15	0.10	0.17	0.15	0.14	0.040	0.64	0.28	0.32
	30	0.78	0.71	0.87	0.63	0.71	0.77	0.76	0.127	0.27	0.19	0.38
	60	0.83	0.91	0.88	0.79	0.87	0.94	0.88	0.084	0.07	0.23	0.65
CA (Eq/L)	0	0.11[a]	0.12[b]	0.12	0.10	0.11	0.11	0.12	0.011	0.03	0.36	0.12
	30	0.15	0.15	0.16	0.15	0.15	0.15	0.15	0.009	0.76	0.60	0.40
	60	0.15	0.16	0.16	0.15	0.15	0.16	0.16	0.009	0.25	0.68	0.13

Different starters resulted from a 2 × 5 design combining or not a *C. boidinii* TOMC-Y13 strain with four strains of *L. pentosus* or any (N). Each starter was applied to 2 different fermenters.

Results are shown by main effects as no significant interaction was found. Mean values shown in the *C. boidinii* columns correspond to 10 fermenters each. Mean values for different *L. pentosus* strains columns correspond to 4 fermenters each.

C: Main effects of including *C. boidinii* TOMC-Y13.

L: Main effects of including different *L. pentosus* strains.

C × L: Interaction effect.

MT & WT. Mutant Type and Wild Type strains, characterized by a different amount of production of EPS (higher in MT).

RSE: Residual standard error.

545 Different superscript letters within a row mean a significant difference ($P< 0.05$) among
546 treatments.

Table 2 Effects of different starters on the microbiological fermentation of table green-olives. Variables included total plate counts of total yeast and lactobacilli, on olive surfaces or liquid brines (log CFU/mL) at Days 0, 30 and 60 of fermentation.

			Main effects										
			C. boidinii		*L. pentosus* strains						*P*-Value		
		day	+	-	119 WT	119-14MT	13B4 WT	13B4-13MT	N	RSE	C	L	C × L
Yeast	olive	0	1.53	0.98	2.26	0.57	1.08	1.55	1.33	0.560	0.36	0.76	0.29
		30	0.37	0.68	ND	0.65	ND	1.04	0.93	1.061	0.54	0.51	0.88
		60	1.92[a]	0.66[b]	0.71	2.36	1.36	0.46	1.57	0.832	0.01	0.06	0.03
	brine	0	3.22	3.52	3.81	2.83	3.39	3.59	3.22	0.742	0.39	0.44	0.38
		30	3.54	3.20	2.32	3.49	3.75	3.52	3.76	1.045	0.48	0.33	0.29
		60	1.90	2.04	1.74	2.00	2.04	2.02	2.04	0.343	0.40	0.70	0.20
Lactobacilli	olive	0	5.54	5.34	5.90	5.47	5.02	6.08	4.96	0.726	0.59	0.30	0.27
		30	8.18	8.07	8.26	8.10	8.33	7.43	8.50	0.477	0.62	0.07	0.17
		60	6.23	6.20	6.28	6.27	5.96	6.06	6.50	0.235	0.74	0.06	0.82
	brine	0	7.54[a]	9.55[b]	7.62	8.86	8.35	8.56	9.34	1.382	0.01	0.53	0.59
		30	7.71	7.70	7.80	7.84	7.56	7.60	7.72	0.466	0.93	0.89	0.87
		60	6.48	6.23	6.31	6.34	6.49	6.37	6.28	0.468	0.27	0.98	0.68

Different starters resulted from a 2 × 5 design combining or not a *C. boidinii* TOMC-Y13 strain with four strains of *L. pentosus* or any (N). Each starter was applied to 2 different fermenters.

Results are shown by main effects. Mean values shown in the *C. boidinii* columns correspond to 10 fermenters each. Mean values for different *L. pentosus* strains columns correspond to 4 fermenters each.

C: Main effects of including *C. boidinii* TOMC-Y13.

L: Main effects of including different *L. pentosus* strains.

C × L: Interaction effect.

MT & WT. Mutant-Type and Wild-Type strains, characterized by a different amount of production of EPS (higher in MT).

RSE: Residual standard error.

563 Different superscript letters within a row mean a significant difference ($P< 0.05$) among
564 treatments.

565 ND. Not determined.

566

Table 3 Effects of different starters on the ability of the EPS isolated from green-olive brines to attach ETEC K88 and interfere with its adhesion to IPEC-J2 cells. Data are expressed as the Δ *t* (hours) observed between the $t_{OD=0.05}$values registered for each EPS sample and its PBS control included in a same assay. Data are expressed as $\Delta t_{OD=0.05}$values related to the control-PBS included in each assay. For details see Material and methods section.

	Main Effects										
	C. boidinii		*L. pentosus* strains						*P*-value		
Test	**+**	**-**	**119 WT**	**119-14MT**	**13B4 WT**	**13B4-13MT**	**N**	**RSE**	**C**	**L**	**C × L**
AT	-0.87	-0.95	-1.10	-0.86	-0.82	-0.93	-0.84	0.476	0.68	0.91	0.60
CT	0.22	0.30	0.20^{ab}	0.47^{a}	0.19^{b}	0.17^{b}	0.29^{ab}	0.088	0.11	0.03	0.05
ET	0.47	0.46	0.57	0.44	0.53	0.41	0.37	0.290	0.97	0.85	0.78
DT	0.08	0.06	0.10	0.04	0.03	0.05	0.12	0.065	0.57	0.30	0.25

Different starters resulted from a 2 × 5 design combining or not a *C. boidinii* TOMC-Y13 strain with four strains of *L. pentosus* or any (N). Each starter was applied to 2 different fermenters. EPS isolated from each fermenter was tested in two independent in vitro assays (each in triplicate).

Results are shown by main effects. Mean values shown in the *C. boidinii* columns correspond to 10 fermenters each. Mean values for different *L. pentosus* strains columns correspond to 4 fermenters each.

C: Main effects of including *C. boidinii* TOMC-Y13.

L: Main effects of including different *L. pentosus* strains.

C × L: Interaction effect.

ETEC K88: Enterotoxigenic *Escherichia coli* K88.

NF-*E. coli*: Non-fimbriated *E. coli*.

RSE: Residual standard error.

MT & WT. Mutant-Type and Wild-Type strains, characterized by a different amount of production of EPS (higher in MT).

AT: Adhesion Tests; CT: Competition Test; ET: Exclusion Test; DT: Displacement Test.

Different superscript letters within a row mean a significant difference ($P< 0.05$) among treatments.

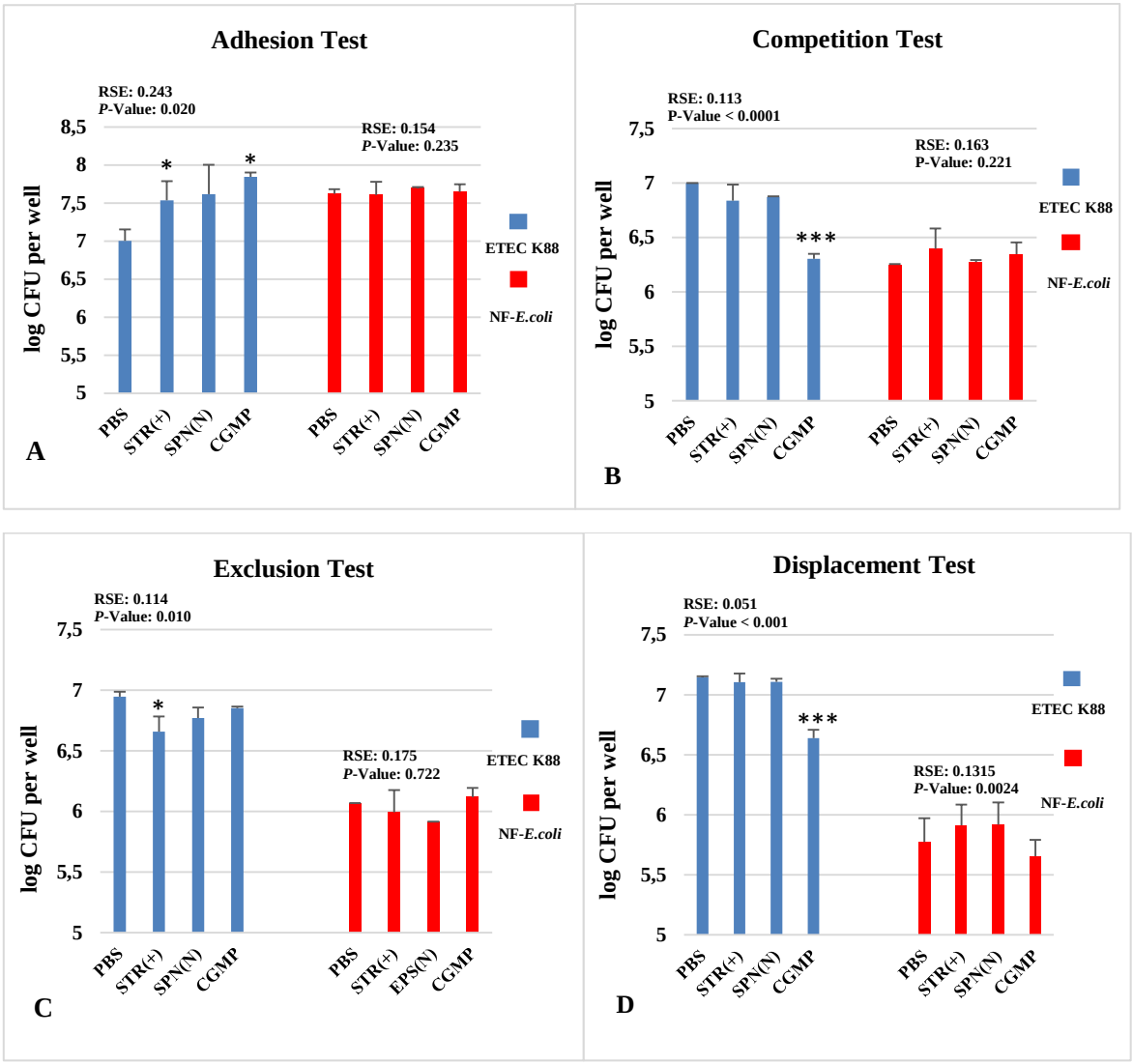

Fig. 1 Ability of exopolysaccharides (EPSs) isolated from green-olive brines to attach ETEC K88 and/or interfere with its adhesion to IPEC-J2 cells. Number of bacteria (log CFU per well) that attached to coated wells (Adhesion Test) or IPEC-J2 cells (Competition, Exclusion and Displacement Tests). Mean values were from two independent *in vitro* assays (each in triplicate) considered as the experimental units. PBS was included as the negative control and casein glycomacropeptide (CGMP) as the positive control. For details in preparation of samples, see Material and methods section.

RSE: Residual standard error.

Superscript asterisks of treatments mean significant differences when compared to negative control (PBS): *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

ETEC K88: Enterotoxigenic *Escherichia coli* K88.

NF-*E. coli*: Non-fimbriated *E. coli*.

STR (+): EPS isolated from the green olives fermented with different starters (n=18).

SPN (N): EPS isolated from green olives fermented spontaneously (n=2).

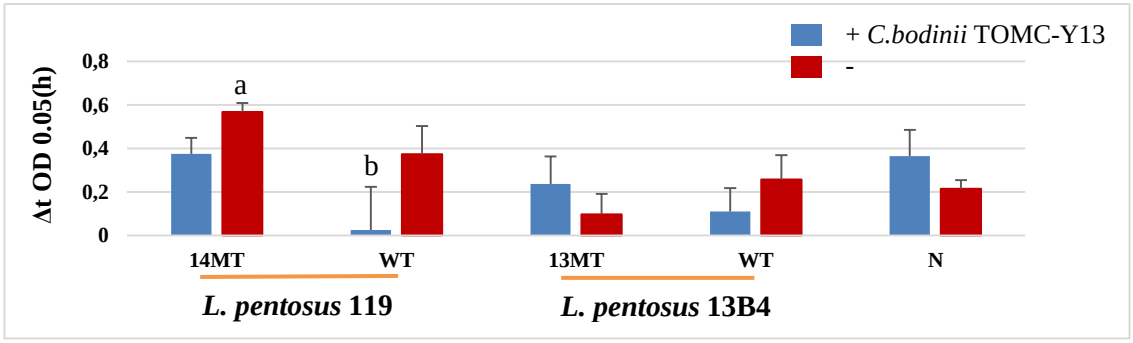

Fig. 2 Effects of different starters on the ability of the EPS isolated from green-olive brines to reduce the adhesion to IPEC-J2 cells. Results from *in vitro* competition test (test ability to block fimbria). Different starters resulted from a 2 × 5 design combining or not a *C. boidinii* TOMC-Y13 strain with four strains of *L. pentosus*. Treatments also included one spontaneous fermentation with no starter. Data are expressed as $\Delta t_{OD=0.05}$values related to the control PBS included in each assay. Mean values came from two independent *in vitro* assays (each in triplicate). For details, see Material and methods section.

MT & WT. Mutant-Type and Wild-Type strains, characterized by a different amount of production of EPS (higher in MT).

Different superscript letters mean a significant difference ($P< 0.05$) among treatments.

619