Exopolysaccharides (EPSs) obtained from green olive brines could reduce the adhesion of enterotoxigenic *E. coli* (ETEC) K88 to porcine intestinal epithelial cells.

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Abstract

This study aims to explore biological functions against enterotoxigenic *E. coli* (ETEC) K88 of the isolated exopolysaccharides (EPSs) produced during the industrial fermentation of olives. Exopolysaccharides were isolated from five industrial fermenters. Analysis of their monosaccharide composition by GLC revealed that the main components were glucose (27%-50%) and galactose (23%-33%) followed by rhamnose (4-23%) and arabinose (6-

17%). The ^1H NMR spectrum showed a very similar profile between samples, and a more in-depth analysis revealed the presence of an α -pyranose in the form of α -D-Glcp-(1 $\rightarrow$) and two different α -furanoses, with chemical shifts values, suggesting the presence of α -D-Glcf and α -D-Galf. Miniaturized *in vitro* tests demonstrated the ability of EPS samples to attach specifically to ETEC K88 ($P < 0.05$) with variable intensity. The competition test, was not able to show ability to block the ETEC K88 adhesion to IPEC-J2 cells, however in the displacement test, all EPS samples were shown to effectively remove the pathogen once attached to the cells ($P < 0.01$). These results suggest that the EPSs produced during the fermentation of table green olives could be able to interfere with the attachment of opportunistic pathogens to the intestinal epithelial cells. This would open the possibility for novel functional properties for this traditional Mediterranean fermented food and for the isolated EPSs as candidates for nutraceuticals to be used in human and/or animal diets in the prevention and treatment of ETEC diarrhoea..

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC), a universal intestinal inhabitant, is one of the main responsible bacteria of diarrhoea in humans and farm animals.^{1,2} Around the world, ETEC causes millions of clinical cases every year, with more than 300,000-500,000 children's deaths annually,³ also being regarded as the ringleader for travelers' diarrhoea.^{4,5} In farm animals, particularly at early stages such as suckling and weaned piglets, ETEC also causes death rates and a decreased growth rate, compromising animal welfare and leading to considerable economic losses.^{6,7} Despite many of the serotypes of ETEC being species-specific, controlling this opportunistic pathogen in farm animals is undoubtedly a key point to control possible zoonosis, and moreover, the spreading of antibiotic resistance among *E. coli*⁸ within the "one health" concept.⁹

Antibiotic resistance has been described as a "global health emergency" by the World Health Organization. A global action plan on antimicrobial resistance was endorsed at the World Health Assembly in May 2015,¹⁰ making a call for investment in the development of new antimicrobial medicines that conserve their effectiveness and longevity. Regarding the use of antimicrobials in agriculture, over the last decade, the scientific community and

industry have been searching for alternative ways to maintain animal health levels and productivity. This has been true particularly in Europe, where the use of antibiotics as growth promoters in livestock was banned in 2006.¹¹ One of the alternative ways to fight pathogens is to target their virulence factors, making them innocuous without exerting selective pressure as antimicrobials do. One of the main virulence attributes of ETEC are adhesins and enterotoxins.⁷ ETEC adhesins primarily attach to the host intestinal tract by their fimbriae, connecting with receptors on cell surfaces; subsequently, enterotoxins secreted by ETEC cause diarrhea, with a net loss of water.^{12,13} Based on this mechanism, blocking or inhibiting ETEC fimbriae or host receptors by exogenous molecules, like biologically active nutrients in human food or animal feed, can be expected to prevent gastrointestinal colonization as an ecological and competition-based strategy.¹⁴

Green table olives are one of the most popular, plant-derived fermented products and a particularly important industry in Mediterranean regions like Spain. The manufacturing process starts with the elimination of oleuropein by diluted lye solution (sodium hydroxide). After washing, green olives are covered with brine in suitable containers, and then the fermentation is carried out for several months.^{15,16} Fermentation occurs spontaneously by the microbiota of the olives although it is also possible to use starter cultures to better control the fermentation process.¹⁷ Lactic acid bacteria (LAB) have been considered to be the main bacteria responsible for the fermentation by transforming sugars into lactic acid.¹⁸ Yeast also plays important roles during the fermentation as they can provide LAB with vitamins, amino acids and purines, and improve LAB growth¹⁹. During the fermentation, some yeast species and *Lactobacillus pentosus* (as the only LAB species) also form a mixed biofilm on the surfaces of olive skin.^{18,20} To build up the biofilm matrix, these microorganisms produce exopolysaccharides (EPSs), and during certain periods of the fermentation process it is possible to isolate important amounts of these polymers of carbohydrates from the brines.

Bacterial exopolysaccharides (EPSs) play an important functional role for the bacteria, but they have also been demonstrated to be behind some beneficial properties of fermented dairy products.²¹ Among other effects, they have been described as being able to interact with specific receptors located on eukaryotic cells and to modulate the cellular

response.^{22,23} Regarding the EPSs produced during the fermentation of table olives, preliminary works of our group have demonstrated that EPS isolated from olive brines could impair the adhesion of ETEC K88 to the porcine intestinal mucus and porcine intestinal epithelial cells (IPEC-J2).^{24,25} However, until now, there are few studies regarding the functional properties of these EPSs isolated from olive brines as a potential nutraceutical or feed additive.

In this study we aim, therefore, to assess the anti-adhesive properties of EPSs obtained from brines of five different industrial olive fermenters. The complex structure and chemical composition of polysaccharides were investigated by HPLC and nuclear magnetic resonance (NMR) spectroscopy. The ability to interfere with ETEC K88 adhesion to IPEC-J2 was evaluated using different, miniaturized *in vitro* models.

2. Materials and Methods

2.1 Brine samples

Brine samples were supplied by JOLCA S.L. (Huevar del Aljarafe; Seville, Spain), and they were obtained from five different, Spanish-style green olive fermentations. Each fermenter consisted of 10,000 kgs of the fruits, Hojiblanca variety, and 6,500 litres of brine (6%, NaCl). After 15 days of brining the olives, samples of brine (1 litre each) were withdrawn and immediately used for EPS purification.

2.2 Exopolysaccharide extraction and purification

The extraction of EPSs from olive-fermentation brines was carried out using the method described by Sanchez et al.²⁶ with some modifications. Briefly brine was firstly centrifuged at 10,000 x g at 4°C for 30 min to get rid of suspended solids and bacteria. Then, 10% w/v trichloroacetic acid (TCA) was added into this fermentation liquid and stirred for 30 min at RT followed by a second centrifugation to remove the precipitate. Two volumes of 96% cold ethanol (4°C) were used to precipitate the EPSs at 4°C overnight. After this, the precipitated EPSs were recovered by centrifugation, re-suspended in ultrapure water and dialysed in dialysis tubes of a 10 kDa-14 kDa molecular-mass cutoff (Medicell Membrane

Ltd.; UK) for 48h at 4°C. Dialysate was subsequently lyophilized and stored at RT until use.

2.3 Monosaccharide composition analysis

The analysis of the constituent's sugars of the EPS samples was carried out by Gas Liquid Chromatography (GLC) following the methodology described by Englyst et al.,²⁷ in which polysaccharides are isolated by precipitation in 80% ethanol, then hydrolysed by sulfuric acid, and the released component sugars are measured as their alditol acetate derivatives.

2.4 NMR analysis

Initially, a ¹H NMR spectrum of each of the EPS samples was performed. Subsequently, and for a more in-depth study, one of the samples (EPS6) was subjected to an additional purification process.

After the TCA treatment, the supernatant obtained was serially filtered to retain the fraction between 300 kDa and 10 kDa. Exopolysaccharides from the dialysate were subsequently precipitated with two volumes of 96% cold ethanol (4 °C) and the sample was freeze-dried. For the NMR analysis, dried EPS samples were dissolved in 600 µL of D₂O (99.96% D, Cortecnet; Voisins-le-Bretonneux, France) and transferred to 5-mm-diameter NMR tubes.

All NMR experiments were recorded on a Bruker Avance II 600 NMR spectrometer (Bruker Biospin; Rheinstetten, Germany) operating at a ¹H NMR frequency of 600.13 and at a temperature of 298.0 K, and equipped with a TBI probe with Z-gradients. ¹H and ¹³C NMR analyses were achieved by the performance of 1D ¹H and 2D NMR (¹H-¹H COSY, ¹H-¹H TOCSY and ¹H-¹³C HSQC) experiments using Bruker standard-pulse sequences and under routine conditions. 2D Diffusion-ordered NMR spectroscopy (DOSY) experiments were performed using a stimulated-echo sequence incorporating bipolar gradient pulses and longitudinal, eddy-currentdelay (BPP-LED).²⁸ A diffusion time of 800 ms and bipolar rectangular gradient pulses of 1.5 ms were employed.²⁹

2.5 Effects of the EPSs brines on ETEC K-88 attachment to IPEC-J2 cells

2.5.1 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 x g for 5min (Mikro 220R, Hettich Zentrifugen). All supernatants were stored at -20°C 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.

2.5.2 *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,³⁰ with serotype (O149:K91;H10(K-88)/LT-I/STb). The other strain was a non-fimbriated *E. coli* (NF-*E. coli*) (F4-, F6-, F18-, LT1-, ST2+, Stx2e-) that was kindly donated by the Departament de Sanitat i d'Anatomia Animals of the Universitat Autònoma de Barcelona, and was isolated from the faeces of a post-weaning piglet. The ETEC K88 strain was grown in unshaken Luria broth (LB) at 37°C for 24h, and the non-fimbriated strain was cultured in shaking LB. Finally, cultured bacteria were centrifuged (1,700 x g, 10 min., 20°C) and the cell pellet was re-suspended in PBS buffer and adjusted to an optical density (OD) of 1 at 650 nm. For the adhesion test, adjusted suspensions were used directly (OD=1); for the *in vitro* test with IPEC-J2 cells, a dilution of 1/100 times of this suspension was performed to reach a final concentration of 6.5-7 log CFU/ml in order to optimize the bacteria/cells ratio.

2.5.3 Cell-culture growth

The IPEC-J2 cells (epithelial cells isolated from the jejunum of the neonatal piglet), kindly donated by Dr. Antony Blikslager, from the College of Veterinary Medicine (North Carolina State University), were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (GIBCO®, Ref.no.:31331-028, Life Technologies; Spain), with insulin, transferrin, selenium and ethanolamine added as ITS solution (GIBCO®, Ref.no.:41400-045, Life Technologies; Spain). Cells were maintained in an incubator at 37°C with an atmosphere of 5% CO₂. Cells were used between passages 95 and 105 and were routinely tested to be free of mycoplasma contamination. For the *in vitro* tests (competition, exclusion and

displacement tests described below), 2×10^5 cells were seeded into 96-well flat-bottom plates (Nunclon Delta Surface, Ref.no.: 167008, Thermo Scientific; Denmark) in a 200 μ l volume. After 24h culture in an atmosphere of 5% CO₂ at 37°C, cell confluence was confirmed under the microscope by a technician specifically trained for this, and then cells were washed once with PBS. Two-hundred-microlitre CO₂-independent medium (GIBCO[®], Ref.no.: 18045-054, Life Technologies; Spain) were added into each well and cells were left in the incubator (37°C for 24h without CO₂) until use.

2.5.4 Adhesion test

Adhesion test (AT) methodology was previously described by González-Ortiz et al.²⁵ The aim of the test is to determine the ability of tested products to specifically attach *E. coli* K88. Briefly, 300 μ l of the PBS soluble extracts (1:100 w/v) obtained from the different EPSs and CGMP (described above) were added into 96-well high-binding polystyrene microtiter plates (Microton F plate 655 092; Greiner Bio-One BV) and incubated at 4°C overnight. Sterile PBS was used to wash the plates to remove non-binding material. Wells were incubated with 1% bovine serum albumin and 0.5% sodium azide in PBS (w/v) at 4°C for 1h to block non-specific adhesion sites. After two washes with sterile PBS, 300 μ l of the bacteria suspensions (ETEC K88 or NF-*E. coli*) were added and incubated for 30 min at room temperature. Wells were washed three times with sterile PBS to remove the non-attached bacteria. Three-hundred-millilitre sterile LB were/ added and the sigmoidal growth of bacteria was measured in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37°C for 12h at a wavelength of 650 nm at 10-minute intervals.³¹ All readings were taken in two independent assays and in triplicate per trial.

2.5.5 Competition Test

The Competition test's (CT) aim is to determine the ability of tested product to block adhesins of bacteria, and was performed as previously described by González-Ortiz et al.²⁴ Briefly, IPEC-J2 cells were first cultured in a CO₂-independent medium in 96-well flat-bottom plates for 24h. PBS soluble extracts (1:100 w/v) were gently mixed with each *E. coli* strain suspension. The mixtures were immediately added to confluent monolayers of IPEC-J2 in a 200 μ l volume. IPEC-J2 cells were incubated with the mixtures at 37°C for 30

min to allow non-blocked bacteria to adhere to cells. Wells were gently washed once by gently pipetting them with sterile PBS to remove the non-adhered bacteria, but without disturbing the cell monolayer. Two-hundred-microlitre CO₂-independent medium was 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°C for 12h at a wavelength of 650 nm at 10-minute intervals. All readings were taken in two independent assays and in triplicate per trial.

2.5.6 Exclusion Test

The Exclusion test's (ET) aim is to determine the ability of the tested products to block receptors in intestinal cells. The procedure is based on methods described by Salcedo et al.,³² but with modifications. Briefly, IPEC-J2 cells were first cultured in a CO₂-independent medium in 96-well flat-bottom plates for 24h at 37°C. One-hundred microlitres of the different EPS and CGMP solutions (1 %) were gently mixed with an equal volume of PBS (final concentration 0.5%). After this dilution, mixtures were immediately added to confluent monolayers of IPEC-J2 in a 200 µl volume. IPEC-J2 cells were incubated with mixtures at 37°C for 30 min to allow EPSs to adhere to cells. Wells were gently washed twice with sterile PBS to remove the non-adhered EPSs, but without disturbing the cell monolayer. One-hundred microlitres of *E. coli* strains bacteria was also mixed with 100 µl of PBS or CGMP solutions, and IPEC-J2 cells were co-incubated with this mixture again at 37°C for 30 min. Cells were washed twice with sterile PBS as before. Two-hundred-microlitre CO₂-independent medium was added to allow for the growth of the adhered bacteria and to keep cells alive. Plates were also covered by a film (VWR, Cat No.6094-064) and monitored at 37°C as the description in CT. All readings were taken in two independent assays and in triplicate per trial.

2.5.7 Displacement Test

The Displacement test's (DT) aim is to test a possible higher affinity of microbial adhesins to attach tested products than to intestinal receptors. Methods have been adapted from Salcedo et al.,³² with modifications. Basically, all of the steps are the same as in the ET. In

this case, IPEC-J2 cells were first co-incubated with 200 μ l of *E. coli* suspensions at 37°C for 30 min. After two PBS washings, IPEC-J2 cells were incubated with 200 μ l of EPS or CGMP solutions (0.5%) at 37°C for 30 min. Cells were washed twice with sterile PBS as before. The remaining steps were the same as in DT. All readings were taken in two independent assays and in triplicate per trial.

2.5.8 Bacterial CFU counts and $t_{OD=0.05}$ correlations

The final OD values ($t_{OD=0.05}$) were translated into colony forming units (CFU) per well that adhered to EPSs (in the AT) or IPEC-J2 cells (in the CT, ET and DT) by correlations previously obtained between OD650 nm values and colony counts of each of the bacterial strains on Luria Agar (LA). 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 μ l per well of each dilution and bacteria were added into microtitration plates in three replicates. Plates monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37°C 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.²⁴

2.5.9 Statistical Analyses

The OD650 data of these four 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.³³ From the time at which the bacterial growth reached an OD650 nm of 0.05 ($t_{OD=0.05}$, h), the log CFU was calculated by using linear models in previous descriptions. Significant differences between treatments in terms of log CFU were determined by a linear model with two-way analysis of variance (ANOVA), with EPS samples as a fixed factor and the replicated assay as a blocking factor using the lm() function stats package from the R v.3.3 free software. Differences between means were tested by the Tukey-Kramer adjustment for multiple comparisons. Values are presented as means $\pm$ SD.

3. Results

3.1 Monosaccharide composition analysis

Percentages of constituent sugars in the different EPS samples are shown in Table 1. Analysis revealed that the percentage of constituent sugar of EPS samples, although showing differences between industrial fermenters, maintained common general characteristics. In all samples, glucose (27%-50%) and galactose (23%-33%) were the main monosaccharides, followed by rhamnose (4%-23%), arabinose (6%-17%) and fucose (1%-3%) (Not detected in sample EPS1). Galacturonic acid was present in a range of 0.1%-1.1%, and glucuronic acids were not detected in any EPS sample.

3.2 NMR analysis

Initially, a ^1H NMR spectrum of each EPS sample was recorded. Spectrums for each sample can be seen in Fig. S1. One of the samples (EPS6) was further submitted to a more in-depth study. The NMR analysis showed the presence of a mixture of both α and β hexose units (Figs. 1 and 2). In the case of α -hexoses, three intense signals in the anomeric proton region of the ^1H - ^{13}C HSQC were observed. Based on their ^1H and ^{13}C chemical shifts, a peak at δ_{H} 5.22 and δ_{C} 94.6 ppm was assigned to α -D-Glcp-(1 $\rightarrow$), with typical lower δ_{C} value than the other two signals due to the pyranose ring nature and a lower δ_{H} value than that of free α -D-Glc,³⁴ because of the substitution at the 1 position. Peaks at δ_{H} 5.28 (δ_{C} 105.4) and δ_{H} 5.12 (δ_{C} 103.5) ppm, corresponding to α -furanoses (5-membered ring hexoses), suggested the presence of α -D-Glcf and α -D-Galf with different substitution patterns. For β -hexoses, a set of unidentified broad signals in the anomeric proton region at δ_{H} 4.67-4.56 ppm and δ_{C} 100.3-99.2 ppm (via ^1H - ^{13}C HSQC correlation) was observed, revealing their presence as β -pyranoses (6-membered ring hexoses).³⁵ Characteristic spin-systems networks corresponding to α and β forms of glucose and galactose residues were observed in the TOCSY spectrum (Fig. S2). Attempts to establish more structural insights failed due to the high complexity of the 4.6-3.2 ppm proton region and also to the broad line shape.

The DOSY NMR experiment is a versatile tool for determining the average molecular weight of linear water-soluble polysaccharides in a simple, fast way and with minimal

sample manipulation. DOSY experiments were carried out on sample EPS6, following the method described by Viel et al.,²⁹ and results obtained were compared to those previously reported.²⁹ The DOSY spectrum of EPS6 showed a diffusion coefficient D ranging from $[2.0-1.3] \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ (Fig. S3), which, according to Viel et al.,²⁹ corresponded to an estimated molecular weight range of around [400-700] k Da for the exopolysaccharides in the sample after purification. This result was in accordance with the purification method.

3.3 Interference of ETEC K88 attachment to IPEC-J2 cell by EPSs

The ability of EPS samples isolated from different olive fermenters to attach ETEC K88 or NF-*E. coli* was tested by the adhesion test (AT), and results are shown in Fig. 3. When incubated with ETEC K88, EPS2 and EPS5 could attach significantly more bacteria than could the negative control (PBS) ($P < 0.05$), reaching similar values as to the positive control (CGMP). Also, sample EPS1 showed a statistical trend to increase the attachment of ETEC K88 ($P = 0.06$).

Results from the Competition test (CT) are shown in Fig. 4. No one of the EPS samples evaluated reduced the attachment of ETEC K88 to IPEC-J2 cells, nor of NF-*E. coli*. Regarding Exclusion test (ET), Fig. 5 shows how EPS2 and EPS3 reduced the attachment of ETEC K88 bacteria to IPEC-J2 cells ($P < 0.05$) significantly, and EPS4 and EPS5 also show a numerical decrease ($P < 0.13$). When evaluated with NF-*E. coli*, ET did not show any effect of EPS samples, as compared to PBS. Regarding the Displacement test (DT) (Fig. 6), all EPS samples removed ETEC K88 efficiently ($P < 0.01$) from the cells. As previously, no differences were found between EPS samples and PBS when incubated with NF-*E. coli*.

4. Discussion

The recognition and attachment to receptors on the surface of epithelial cell is a first step of pathogenic ETEC to colonize the host intestinal tract. This recognition is mediated by *E. coli* fimbriae (also known as adhesins), particularly fimbria F4 in the case of ETEC K88.^{12,13} On porcine intestinal epithelial cells, carbohydrates are generally recognized as the receptor structures for ETEC K88 adhesins,^{36,37} glycoproteins and glycosphingolipids

being the most common intestinal glycoconjugate receptors.³⁸⁻⁴⁰

Bacterial EPSs are carbohydrate polymers covering the bacteria surface, offering protection to bacteria and helping them to adapt to different media. Often, these capsules serve as adherents of cells to surfaces and may be overproduced to become carbohydrate reserves for subsequent metabolism.⁴¹ Due to their different properties and functionalities, in the last few years, different industrial applications have been found for large-scale produced EPSs like dextran or alginate.⁴¹ Together with their technological properties, bacterial EPSs have been suggested to offer potential health benefits based on the ability of some EPSs to attach to intestinal receptors, to block bacteria adhesins or to inactivate toxins.⁴²⁻⁴⁴

The fermentation of green olives is a complex process that has been previously described¹⁶ in which hundreds of bacteria and yeast participate.⁴⁵ Among all of the microorganisms involved, *Lactobacillus pentosus* has been described as one of the main actors able to produce, together with the yeast population, complex biofilms on the olive epidermis based on the production of EPS.^{20,46} It has been hypothesized that these EPSs probably help microorganisms to survive for a long time, resist environmental stresses, and embed bacteria on the olive surfaces to obtain a high concentration of nutrients.^{20,46}

Although in most of the cases the fermentation of the olives is not controlled and is carried out spontaneously by the native microbes that inhabit the olives, the fermentation process has been found to follow a general common pattern.^{15,16} Despite this, it is obvious that there can be variations, depending on the course of the fermentation, that could affect the nature of the EPSs obtained during the industrial process. This natural variability could be the reason for the differences found between EPS samples tested in this work, as they were isolated from different industrial fermenters.

4.1 Variability in chemical structure

Regarding the monosaccharide composition of our EPS samples, a certain variability could be seen between batches, but a quite consistent composition in terms of galactose and glucose as the two main components. In this regard, results from the ¹H NMR spectrum (Fig. S1) also revealed a pretty similar profile between batches. To our knowledge, this is

the first time that EPS isolated from green-olive brines has been characterized in these terms. Other groups had characterized the oligosaccharide composition of EPS produced by pure cultures of *Lactobacillus pentosus* isolated from green-olive brines.²⁶ In that study, they identified two different EPSs mainly composed of glucose (Glu) and rhamnose (Rha) (EPS A: 1.9×10^6 Da), and glucose and mannose (EPS B: 3.3×10^4 Da), and they also showed that culture conditions have a clear influence on the ratio and concentration of each EPS. The subsequent research showed D-glucose, D-glucuronic acid and L-rhamnose of EPS A in a molar ratio of 1:2:2.⁴⁷ From our results, the high contents of galactose and the putative molecular weight between 400 kDa-700 kDa estimated by the DOSY experiments suggest that the EPSs isolated from the olives brines are different from those described for *L. pentosus*. It is probable that other bacteria or yeast could have been involved in the production of EPSs in the brine. A more in-depth analysis by NMR spectroscopy revealed the presence of α and β hexoses. Among the α hexoses, an α pyranose in the form of α -D-Glcp-(1 $\rightarrow$) and two different α -furanoses, with chemical-shift values suggesting the presence of α -D-Glcf and α -D-Galf, were observed (Figs. 1 and 2). Other microbial exopolysaccharides have been reported to include α -furanose rings similarly to our study. In this regard, the review of Broadbent et al.⁴⁸ describes, for *Streptococcus thermophilus* EPS, different basic repeating structures, including pyranose and furanose configurations for glucose, galatose and rhamnose.

4.2 Interference with ETEC-K88 adhesions

In the adhesion test, we were able to demonstrate that two of the five EPS isolates were able to significantly attach ETEC K88, and two other samples were also found to numerically increase the number of attached bacteria, as compared to PBS. In previous works of our group, we were also able to demonstrate attaching properties for other EPS samples²⁴ obtained from other annual harvests. From these results, it could be said that in most of the cases, EPSs obtained from olive brines showed the ability to attach selectively ETEC K88 although this activity could be of variable intensity, depending on the fermentation conditions. In this regard, our group recently performed a study trying to control the spontaneous fermentation of green olives with the introduction of different lactobacilli and/or *Saccharomyces spp.* inocula. In this work, it was found that certain

inocula were able to increase the functionality of the obtained EPSs,⁴⁸ opening the possibility of controlling the fermentation process of table olives for higher functionality.

Regarding the competition test, this gives us an idea of the ability of the tested products to block the ETEC K88 adhesins involved in the recognition of epithelial cell receptors. In our study, tested EPSs did not reduce the number of ETEC K88 attaching to IPEC-J2 cells, suggesting that adhesins involved in the AT were not the same as those involved in epithelial adhesion. Previous works of our group, however, could demonstrate blocking activities of other EPS isolates against IPEC-J2 cells,²⁴ but not intestinal mucus,²⁵ suggesting that blocking activities could depend on variations in the fermentation conditions of the olives. In addition to this, the possible limitations on the method should not be discarded. For example, it should be considered that, in this work, mixtures of EPS extracts and ETEC K88 were immediately exposed to the cells, and if recognition of EPSs by the fimbria is a time-dependent process, variations in time previous to incubation could explain this variability.

Another way by which EPSs could also interfere with the adhesion of ETEC K88 to intestinal cells could be by recognizing and blocking receptors sites. In that sense, it has been published how different high-EPS-producer probiotic strains can improve their adhesion to Caco-2 cells, as compared to the non-mutant strains, diminishing *E. coli* colonization.^{49, 50} Similarly, it has also been described how pathogens like *Vibrio vulnificus*, which lacks/when they lack genes responsible for EPS production, showed lower cyto-adherence to human cells than did the wild strain.⁵¹ Our results from the exclusion test, where IPEC-J2 cells are first incubated with EPS samples, showed that all the EPSs numerically reduced the number of ETEC K88 attached, although only for EPS2, EPS3 were reductions statistically significant. Although a net increase in the adherence of the bacteria to the cells with EPSs in the ET was never detected, the possibility should not be discarded that in some cases EPSs could be acting as an anchor between the ETEC-K88 and the intestinal receptors, depending on the spatial exposure of the different receptor sites on the EPSs. In that sense, some authors have described increments in the adhesion of *Salmonella* Typhimurium and *Clostridium difficile* to intestinal mucus by different EPSs produced by probiotic strains⁴² that the authors relate to the ability of the pathogens to bind

EPSs and of the bound EPSs to adhere to mucus. In another study,⁵² EPSs isolated from *Streptococcus thermophilus* CRL1190 were found *in vitro* to form a bio-adhesive polymer layer on the surface of human gastric adenocarcinoma epithelial cells able to bind *Helicobacter pylori*.

The most relevant results of this study came from the displacement test. In this test, it could be demonstrated that all the tested EPS samples could consistently remove adhered ETEC K88 from IPEC-J2. Removed cells did not reach the levels shown by CGMP as positive control did, but rather intermediate levels. These results would suggest a higher affinity of the ETEC K88 adhesins for EPSs than for IPEC-J2 cell receptors. In this regard, it has been published how ETEC K88 adhesins can bind to β -linked galactose⁵³ and β 1-linked galactose residues forming part of intestinal glycoconjugate receptors.⁵⁴ As shown in Table 1, in the EPS samples tested in this study, galactose was one of the main constituents representing between 23% to 33% of total sugars. These results would suggest that EPSs from olive brines possibly offer galactose residuals to ETEC K88 for their adhesion. In this sense, other studies with an EPS-producing strain of *Lactobacillus crispatus* could demonstrate how this strain could efficiently remove the attached *Candida albicans* from vaginal epithelial cells in displacement assays. When the same authors tested the purified PBS, they also demonstrated how this mannan-rich EPS could reduce invading *C. albicans* in displacement assay although levels of reduction were much higher for the exclusion test, suggesting that the displacement activity in this case was due to a blockage of the intestinal receptors.⁵⁵

In this study CGMP was chosen as a positive control, as we had demonstrated its ability to reduce *in vivo* ETEC K88 attachment to pig intestinal mucosa and to epithelial cells in the competition test in previous works of our group.^{14, 24, 56} Results from CT and DT in this study clearly confirm, once again, the potential of this milk derivative to fight ETEC. However, it failed to reduce pathogen adhesion in ET, suggesting that its mechanism of action is mainly based on the recognition and blockage of bacterial adhesins. From these results, it seems that the glucans from CGMP would play a role as a bacterial receptor analogue for ETEC K88.

5. Conclusions

This study gives a first insight into the chemical structure of the EPSs produced during the industrial fermentation of green olives. Despite certain variability found among different industrial batches, a common pattern has been found with similar ^1H NMR spectrums, α and β forms of glucose and galactose being the main component sugars in all of them. Data from *in vitro* models demonstrated the potential of these EPSs to specifically attach ETEC K88 and to remove the pathogen from IPEC-J2. The mechanism by which this detachment is achieved has not been fully demonstrated, but EPSs could be blocking intestinal receptors sites and/or bacterial adhesins. These results open the possibility for novel functional properties for this traditional Mediterranean fermented food and for the isolated EPSs as candidate for nutraceuticals to be used in human and/or animal diets in the prevention and treatment of ETEC diarrhoea. Further research is needed to elucidate the possible anti-adhesive properties of these EPSs in *in vivo* models and to determine the optimum fermentation conditions of green olives.

Conflict of interest

There are no conflicts to declare.

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Fig. 1 1D ^1H NMR spectrum of sample EPS6 with the anomeric region expanded. Spectrum acquired at a magnetic field of 600 MHz and at 298.0 K.

Fig. 2 Expanded anomeric region of the ^1H - ^{13}C HSQC spectrum of sample EPS6. Spectrum acquired at a magnetic field of 600 MHz and at 298.0 K.

Fig. 3 Adhesion test (AT): Number of bacteria (log CFU per well) that attached to wells coated with EPS samples isolated from different olive fermenters. Mean values (log CFU per well $\pm$ SD) were from three replicates of two independent *in vitro* assays. A single assay was considered the experimental unit. Tested extracts were equivalent to 1% (w/v) concentration. PBS was included as the negative control and casein glycomacropeptide (CGMP) as the positive control. Fig. 3A shows results with ETEC K88 bacteria, and Fig. 3B with non-fimbriated (NF) *E. coli*. Superscript asterisks of treatments mean significant differences when compared to PBS: T: $P=0.06$; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Fig. 4 Competition test (CT): Number of bacteria (log CFU per well) that attached to IPEC-J2 cells after being previously co-incubated with EPS samples isolated from different olive fermenters. Mean values (log CFU per well $\pm$ SD) were from three replicates of two independent *in vitro* assays. A single assay was considered the experimental unit. Tested extracts were equivalent to 1% (w/V) concentration. PBS was included as the negative control and casein glycomacropeptide (CGMP) as the positive control. PBS1 was the control for EPS1, and PBS2 was the control for the rest of the samples. Fig. 4A shows results with ETEC K88 bacteria, and Fig. 4B with non-fimbriated (NF) *E. coli*. Superscript asterisks of treatments mean significant differences when compared to negative control: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Fig. 5 Exclusion test (ET): Number of bacteria (log CFU per well) that adhered to IPEC-J2 that were previously incubated with EPS samples isolated from different olive fermenters. Mean values (log CFU per well $\pm$ SD) were from three replicates of two independent *in vitro* assays. A single assay was considered the experimental unit. Tested extracts were equivalent to 1% (w/v) concentration. PBS was included as the negative control and casein glycomacropeptide (CGMP) as an additional control. Fig. 5A shows results with ETEC K88 bacteria, and Fig. 5B with non-fimbriated (NF) *E. coli*. Superscript asterisks of treatments mean significant differences when compared to PBS: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Fig. 6 Displacement test (DT): Number of bacteria (log CFU per well) remaining adhered to IPEC-J2 cells after being incubated with EPSs. Mean values (log CFU per well $\pm$ SD) were from three replicates of two independent *in vitro* assays. A single assay was considered the experimental unit. Tested extracts were equivalent to 1% (w/v) concentration. PBS was included as the negative control and casein glycomacropeptide (CGMP) as the positive control. Fig. 6A shows results with ETEC K88 bacteria, and Fig. 6B with non-fimbriated (NF) *E. coli*. Superscript asterisks of treatments mean significant differences when compared to PBS: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1 Constituent sugars of olive brine exopolysaccharide (EPS) samples analysed by GLC (% , w/w)

	Rha	Fuc	Ara	Xyl	Man	Gal	Glu	GalA
EPS1	4.24	ND	9.71	1.25	1.83	32.74	49.45	1.08
EPS2	18.69	2.25	16.83	2.22	1.99	30.43	26.91	0.68
EPS3	18.44	1.90	10.95	2.27	1.45	29.17	35.38	0.66
EPS4	13.69	3.18	17.40	3.11	3.03	32.16	26.31	1.12
EPS5	22.74	1.26	5.81	2.17	1.38	22.96	43.36	0.32
EPS6	2.20	ND	12.14	0.80	0.21	46.20	38.33	0.14
Mean	13.33	1.43	12.14	1.97	1.65	32.28	36.62	0.67
SEM	3.42	0.52	1.80	0.34	0.38	3.13	3.72	0.16

ND: non-detected; SEM: standard error of mean.

Abbreviation: rha: rhamnose; fuc: fucose; ara:arabinose; xyl: xylose; man: mannose; gal: galactose; glu: glucose;; GalA: Galacturonic acid.

Glucuronic acid (GlcA) was always below the limit of detection.

The EPS6 sample corresponds to the sample used for 2D-NMR characterization. It was a sample serially filtered for a molecular weight selection of between 300kDa and 10,000 kDa (See text for details).

Fig. S1 1D ^1H NMR spectrum of samples EPS1 to EPS6 (from top to bottom). Spectrum acquired at a magnetic field of 600 MHz and at 298.0 K.

Fig. S2 ^1H - ^1H TOCSY spectrum of sample EPS6 acquired at a magnetic field of 600 MHz and at 298.0 K.

Fig. S3 2D DOSY spectrum of sample EPS6 acquired at a magnetic field of 600 MHz and at 298.0 K.

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