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# Exopolysaccharide produced by *Weissella confusa*: Chemical characterisation, rheology and bioactivity

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ABSTRACT

The purpose of this study was to characterise the production of exopolysaccharide (EPS) by indigenous lactic acid bacteria (LAB) isolated from traditional Algerian dairy products and to evaluate their possible use in agri-foods. Among the collection of isolated strains, the strain *Weissella confusa* (W4) was selected for its ability to produce EPS once exposed to a sucrose culture medium. EPS produced were first isolated with a standardised method and further characterised in terms of molecular size, antioxidant activity, and rheological properties. Its direct implication in the texture and syneresis of acid milk gel was evaluated offering interesting industrial applications for its use during processes dealing with dairy products.



## 1. Introduction

Dairy products resulting from traditional preparations are considered organic foods (without preservatives or additives) in which native flora with putative potential are attracting the interest of microbiologists and the agri-food sector.

Klila is a traditional fresh or hardened cheese that can be dehydrated from cow, sheep or goat milk curd following heating, cutting and sun exposure. Its manufacture is based on the spontaneous fermentation of milk by its native flora for 48 h at room temperature. In Algeria, Klila is mainly made according to traditional methods at home for family consumption. Consequently, the product is not subject to any control relating to quality and safety. However, for centuries the Algerian population has considered it beneficial for health and even for therapeutic purposes (Boubekri & Ohta, 1996). Many studies have focused on the characterisation of the physico-chemical and microbiological qualities of Klila cheese (Benamara, Gemelas, Ibri, Moussa-Boudjemaa, & Demarigny, 2016; Benlahcen, Mahamedi, Djellid, Sadeki, & Kihal, 2017; Guetouache & Guessas, 2015). This product remains devoid of pathogenic microorganisms, since its manufacture includes a heat treatment, a low pH (around 4.63), an abundance of protective flora and a low value of water activity (Benlahcen et al., 2017).

The fermentation of milk primarily involves lactic acid bacteria (LAB). Some of these bacteria can excrete exopolymers, mainly in the form of exopolysaccharides (EPS) consisting of long chains repeating units of simple sugars that can be associated with branched carbohydrate derivatives. Due to their composition they can be classified as homopolysaccharides (HoPS) or heteropolysaccharides (HePS) (Allison & Sutherland, 1987; De Vuyst & Degeest, 1999; Zeidan et al., 2017), and may be associated with the bacterial cell wall (capsular EPS) or secreted directly into the medium (free EPS) (Pachekrepapol, Lucey, Gong, Naran, & Azadi, 2017). In some cases, bacteria can produce both forms of EPS (Hassan, 2008).

Many roles have been proposed for bacterial polymeric substances that are mainly composed of polysaccharides, proteins, nucleic acids and lipids (Flemming & Wingender, 2010). First, EPS production may be a direct and logical response to selective environmental pressures and is fully dependent on medium composition (Donot, Fontana, Baccou, & Schorr-Galindo, 2012; Mende, Rohm, & Jaros, 2016; Patel & Prajapat, 2013). In a natural environment with harsh conditions, EPS gives cells a competitive advantage and allows them to survive. For example, EPS interacts with ions that can participate in the transport of solutes in

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microorganisms, thus playing a role in adsorption and exchange (Donot et al., 2012). The capacity to coat cells with an EPS layer may protect them from desiccation. Second, EPS play a key role in biofilm formation (Flemming & Wingender, 2010), they ensure the mechanical stability of the biofilm and are involved in the adhesion of bacteria to surfaces, forming ordered assemblies of polymers that surround the colony (Flemming & Wingender, 2001; Ruas-Madiedo, Hugenholtz, & Zoon, 2002).

EPS play a very important role in the dairy industry, mainly in fermented dairy products, such as yoghurt, cheese and dairy-based desserts. In addition, regarding fermented bakery products, many studies have pointed out the stabilising, gelling and thickening properties of EPS of acid lactic bacteria like *Lactobacillus helveticus* and *Streptococcus thermophilus* (Lin & Chien, 2007; Lynch, Zannini, Coffey, & Arendt, 2018). In situ production of polysaccharides has the advantage of improving the textural properties of fermented dairy and cereal products, leading to the reduction or replacement of added ingredients and stabilisers used for this purpose, such as milk proteins, starch, pectin and other hydrocolloids. Ultimately, replacing these additives provides a quality label and lower production costs (Zeidan et al., 2017). EPS have mainly been beneficial for solving syneresis problems in yoghurt and cheese (Jaros, Haque, Kneifel, & Rohm, 2002; Perry, McMahon, & Oberg, 1997). They do not only improve organoleptic behaviour (texture, viscosity), but also allow producing foods with nutraceutical properties (immunomodulatory, protection against gastric ulcers, improvement of digestive transit, hypocholesterolaemic activity, antiviral, antitumour) (Ruas-Madiedo, Salazar, & Clara, 2009), thus they respond to very high consumer demand for food without food additives.

In this context, we focused on characterising EPS produced by a native lactic acid bacterium isolated from an Algerian milk product (Klila) and on its potential role in the dairy industry. Particular attention was given to the presence or not of proteins embedded in the EPS.

## 2. Materials and methods

### 2.1. Bacterial strain and growth conditions

The strain used in this study was isolated from a traditional Algerian cheese (Klila). It was isolated using agar plates containing MRS medium (De Man, Rogosa, & Sharpe, 1960) incubated at 30 °C for 48 h under aerobic conditions. The 16S rDNA gene was amplified by PCR and sequenced Genewize (Paris, France), as described previously (Rousseaux, Hartmann, & Soulas, 2001). These bacteria were identified as *Weissella confusa* and encoded W4. The strain was routinely grown at 30 °C in MRS medium Conda (Madrid, Spain) at pH 5.8 under aerobic conditions.

### 2.2. Detection of EPS production at colony level

The production of increased quantities of EPS at the colony level was tested (Kim, Seo, Hwang, Lee, & Park, 2008). Briefly, the culture was isolated on agar MRS (Conda) pH 5.8 containing different carbon sources (50 g $L^{-1}$): glucose, sucrose, lactose, galactose, mannose or fructose Biochem Chemopharma (Cosne-Cours-sur-Loire, France). After 72 h at 30 °C, sticky or normal appearances of colonies were checked.

### 2.3. Extraction of EPS from isolated acid lactic bacteria

EPS isolation was carried out by ethanol precipitation (Cerning et al., 1994), with few modifications. The W4 strain was grown at 30 °C under aerobic conditions on 1000 mL of MRS medium supplemented with 100 g $L^{-1}$ sucrose. After 48 h, bacteria were removed from the medium by centrifugation at 7000×*g* for 10 min at 4 °C, the clear supernatant was collected and the EPS precipitated by adding three volumes of cold absolute ethanol (Sigma–Aldrich) and kept overnight at 4 °C. The precipitate was recovered by centrifugation at 10,000×*g* for 20 min, followed by a freeze-drying step leading to EPS W4. The final precipitate was resuspended in 10% trichloroacetic acid Sigma-Aldrich (Saint Louis, Missouri, USA), incubated at 4 °C for 30 min and then centrifuged at 4 °C, 10,000×*g* for 20 min to remove the proteins denatured by trichloroacetic acid (TCA). The supernatant was then dissolved in ultrapure water and dialysed for three days against ultrapure water (changed twice each day) using a 10 kDa cut-off cellulose dialysis membrane Thermo Scientific (Runcorn, Cheshire, England). After dialysis, the solution was frozen at −80 °C and freeze-dried Freezone (LabConco Corporation, Kansas, USA) at −50 °C for two days to completely remove the solvent. The obtained powder was designated EPS W4*.

### 2.4. Physico-chemical composition of EPS

Solutions of 1 g $L^{-1}$ of freeze dried EPS were solubilised in ultrapure water Millipore (Merck, Darmstadt, Germany) to quantify neutral and acidic carbohydrates using the phenol and carbazol sulphuric acid method (Usseglio-Tomasset & Castino, 1975). Briefly, a calibration curve [Absorbance = f (sugar concentration)] was performed with pure solutions of glucose or galacturonic acid to quantify neutral and acid sugars, respectively, at 475 nm and 525 nm. The value of absorbance of EPS was then converted via these curves to sugar quantities and expressed in percentage of the initial solubilised EPS matter.

The proteins embedded in the EPS are quantified and expressed in percentage of the initial solubilised EPS matter using the Bradford method (Bradford, 1976).

#### 2.4.1. Estimation of EPS molecular mass

The molecular mass of the EPS was determined by size exclusion chromatography. Freeze-dried EPS was solubilised in ultrapure water at a concentration of 1 g $L^{-1}$ and a volume of 100 µL was injected into a gel filtration chromatographic column of TSKgel (Tosoh, Tokyo, Japan) G5000 PWXL 7.8 mm ID, 30 cm L, kept at 27 °C, eluted with $NaNO_3$ 100 mmoL $L^{-1}$ at 0.7 mL $min^{-1}$, and detected by a Spectra Systems (Thermo Fischer Scientific, Waltham, USA) RI-150 refractometer. The molecular mass was calculated following the calibration curve obtained by using various standard dextrans (1000, 500, 650, 50 kDa, Sigma Aldrich).

#### 2.4.2. IR analysis of EPS: fourier–transform infrared spectra

FTIR experiments were performed in the attenuated total reflection (ATR) mode using a Thermo Scientific Nicolet iS5 and a diamond crystal. Each freeze-dried EPS (approximately 10 mg) was gently placed on the surface of the diamond and pressed with a constant force with a torque screwdriver. Each spectrum was recorded from 500 to 4000 $cm^{-1}$ with a 4 $cm^{-1}$ resolution. The result was an accumulation of 32 scans.

### 2.5. EPS functional properties

#### 2.5.1. Antioxidant activity (scavenging of hydroxyl radical)

The hydroxyl radical scavenging effect of EPS W4 with and without the protein fraction was measured by initiating a Fenton reaction in the presence of EPS and a radical trapping agent, i.e., brilliant green (Sigma–Aldrich). The methodology was adapted from previous studies (He, Luo, Cao, & Cui, 2004; Zhang et al., 2013). Briefly, the reaction mixture containing 1.0 mL of brilliant green

(0.435 mmoL $L^{-1}$), 2.0 mL of $FeSO_4$ (0.5 mmol $L^{-1}$), 1.5 mL of $H_2O_2$ (3.0%, w/v), and 1 mL of EPS samples at various concentrations (1–4 mg $mL^{-1}$) solubilised in distilled water was incubated at room temperature for 20 min, and the absorbance was measured at 624 nm. Hydroxyl radical scavenging activity (HRSA, %) was evaluated by the change in absorbance value after 20 min reaction in the presence ($A_S$) and in the absence of EPS ($A_0$), and with an initial concentration of brilliant green measured by its absorbance (A) and calculated as follows:

$$HRSA\ (\%) = [As - A_0]/(A - A_0)] \times 100$$

### 2.5.2. Rheological and physical measurements of acidified milk

*2.5.2.1. Determination of the rheological behaviour of EPS W4.* The lyophilised EPS W4 was dissolved in ultrapure water at a concentration of 1 g $L^{-1}$. Visco-elastic measurements were performed at room temperature using a stress-controlled dynamic rheometer (MCR 302 Anton Paar, (Graz, Austria)) equipped with parallel plates geometry (diameter of 25 mm, 1 mm gap) and controlled by the Rheocompass (Anton Paar, Graz, Austria). A large oscillation strain sweep (from 0 to 20%) was first performed on the sample to determine the linear viscoelastic response of EPS performed at a constant angular frequency of 1 rad $s^{-1}$. Then, at a constant strain of 1%, the storage modulus (G′) and the loss modulus (G″) were recorded as functions of angular frequency (ω) from 0.1 to 100 rad $s^{-1}$ to evaluate the rheological properties of the EPS structures.

*2.5.2.2. Texture modification of acidified milk by EPS.* To evaluate the impact of the rheological behaviour of EPS W4 on the texture of the acidified milk, whole cow milk was used (Carrefour, France) for three different productions: (i) Milk + gluconodeltalactone (GDL), (ii) Milk + GDL + dextran (1%), (iii) Milk + GDL + EPS (1%).

These three productions were performed using systematically a batch of 400 mL of milk acidified with 2.5% (w/w) GDL (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Just after GDL introduction, milk was put into cups of 40 mL, leading to a yoghurt sample height of 40 mm ($H_0$). Slow acidification was carried out at 42 °C for 40 min until the pH stabilised at 4.6 (to simulate the pH value of yoghurt). The cups were stored at 4 °C before texture analysis (Gentès, St-Gelais, & Turgeon, 2011).

The yoghurts prepared were investigated by TA-XT2 texture analyser (Texture Technology Corp., Scarsdale, NY, USA), as described by Hess, Roberts, and Ziegler (1997). The inoculated yoghurt was analysed using a cylindrical probe, with a base diameter of 3 cm and a height of 4 cm. Penetration speed was constant at 1 mm $s^{-1}$ and the penetration depth (ΔH) was 5 mm corresponding to a strain ratio of 8% ($\Delta H/H_0{*}100$). The maximum force required to penetrate the gel was taken as a measure of the relative gel strength. Analyses were conducted at 4 °C on eight biological repetitions for each production, and each production was tested in triplicate.

A centrifugation method was used to evaluate the induced syneresis of the acidified milk. Thirty grams of each pot was centrifuged (220×*g* for 15 min at 4 °C), the percentage of syneresis was calculated by dividing the weight of the clear supernatant obtained after centrifugation to the total weight of the yoghurt multiplied by 100 (Keogh & O'Kennedy, 1998). Each production and experiment was also tested in triplicate.

### 2.6. Statistical analysis

Each experiment was carried out in triplicate. Student t-test ($n = 3$; $P < 0.05$) was used to investigate the significance of differences between (i) the EPS W4 contents in acidic sugars, neutral sugars and proteins, (ii) the antioxidant effect observed with EPS and (iii) the syneresis and maximum force reached at gel compression in absence and presence of EPS W4.

## 3. Results

Lactic acid bacteria strains isolated from an Algerian cheese (Klila) were characterised for their capacity to produce EPS under culture conditions with high sucrose concentration (50 g $L^{-1}$). Among the twenty strains isolated, one was characterised in more detail since it had the highest capacity to produce EPS, which resulted in the production of sticky colonies using certain sugars as carbon source. Sequencing of the 16S DNA region allowed us to identify this strain as *W. confusa* (W4). This strain was cultivated in MRS containing 50 g $L^{-1}$ of sucrose to promote the production of exopolysaccharides. This activity was evidenced by macroscopic observation of sticky colonies (Fig. 1A) on agar plates. As observed in Fig. 1B, no production of EPS occurred when strain W4 was cultivated using other sugars such as glucose, lactose, galactose, mannose, or fructose as unique carbon sources.

### 3.1. Chemical characterisation of exopolysaccharides

The overall characterisation of the exopolysaccharide fraction (Fig. 2A) highlighted a difference in the amount of neutral and

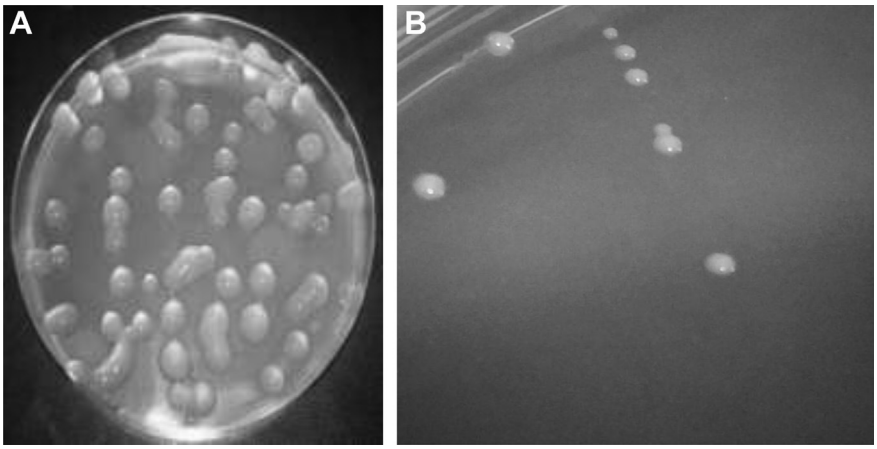


**Fig. 1.** Macroscopic observation of W4 strain colonies grown on agar MRS enriched in sucrose (A) and on agar MRS enriched in glucose (B).

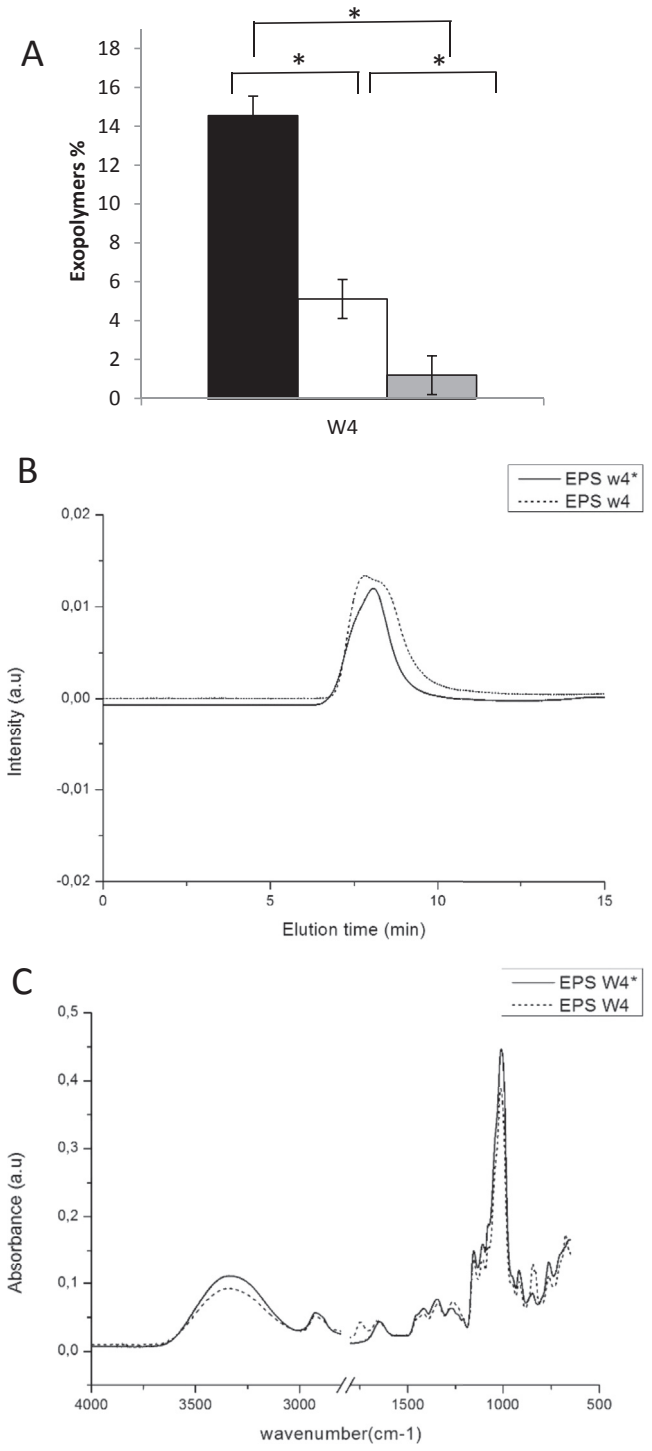


**Fig. 2.** Overall characterisation (A) of isolated EPS W4, neutral polysaccharides (■), acid polysaccharides (□) and proteins (▩), (B) chromatograms of EPS W4 and EPS W4* solubilized at 1 g $L^{-1}$ in ultrapure water, based on a refractive index detector and (C) infrared absorption spectrum of freeze dried EPS W4 and EPS W4*.

acidic sugars. Indeed, the amount of neutral sugars was significantly larger than acidic sugars. Despite protein precipitation with TCA, around 1.5% of the sample was composed of proteins. This can be explained by the quite considerable overlapping of proteins and polysaccharides on EPS W4. This organisation of the EPS structure (network of sugars in which proteins are embedded) may prevent the full precipitation of proteins. However, the elimination of proteins led to a change in the distribution of molecular masses and a modification of the structure of the molecular network (Fig. 2A and B). Moreover, the infrared spectra of EPS W4 and W4* had approximately a similar profile (shown in Fig. 2C), displaying a characteristic broad stretching intense peak around 3405.36 $cm^{-1}$ representing hydroxyl groups and a low stretching band C–H at 2925.06 $cm^{-1}$ whereas the bands in the region of 1744 $cm^{-1}$, 1640 $cm^{-1}$, 1145 $cm^{-1}$, 1016 $cm^{-1}$ and 915 $cm^{-1}$ were due to the protein (N–H) C–O–C glycosidic band vibrations, the glycoside band α(1–6) and the α-glycosidic bond, respectively. However, there was an increase in the hydroxyl group intensity peak and a significant decrease in the protein intensity peak for the EPS W4* obtained after trichloroacetic acid precipitation. According to the calibration curve using standard dextrans, both EPS W4 and EPS W4* had a molecular mass of approximately $10^7$ Da (Fig. 2C).

### 3.2. Functional properties of EPS produced by Weissella confusa W4

#### 3.2.1. Antioxidant activity

Since the hydroxyl radical is the most reactive oxygen, it can cause severe damage due to its capacity to react with all biomacromolecules in living cells (Gülçin, 2006). The antioxidant activity of EPS (W4 and W4*) was evaluated by reducing the bleaching of the brilliant green initially bleached by the Fenton reaction (Fig. 3). The ability of EPS W4 to scavenge hydroxyl radicals was investigated using the Fenton reaction by comparing it with that of ascorbic acid. As shown in Fig. 3, EPS W4 and ascorbic acid exhibited scavenging activities against hydroxyl radicals. EPS W4 could scavenge hydroxyl radicals at concentrations between 1.0 and 4.0 mg $mL^{-1}$, reflecting its antioxidant activity, even if at these concentrations, the scavenging activity was lower than that of ascorbic acid. However, removing proteins from the EPS fraction led to a decrease in the antioxidant activity from 74.3% to 54.6% at 1 mg $mL^{-1}$ and from 82.4% to 63.8% at 4 mg $mL^{-1}$, respectively, for EPS W4 and EPS W4*. These results suggest that the macromolecular structures of EPS were differentially prone to hydroxyl radical attacks and that their reactivity was highly dependent on its protein content and associations with the polysaccharidic fraction.

#### 3.2.2. Determination of the rheological behaviour of EPS W4 and physical measurements of acidified milk

To understand EPS reactivity and mechanical behaviour in dairy products it is essential to know their physico-chemical characteristics and their rheological behaviour in aqueous solution. The

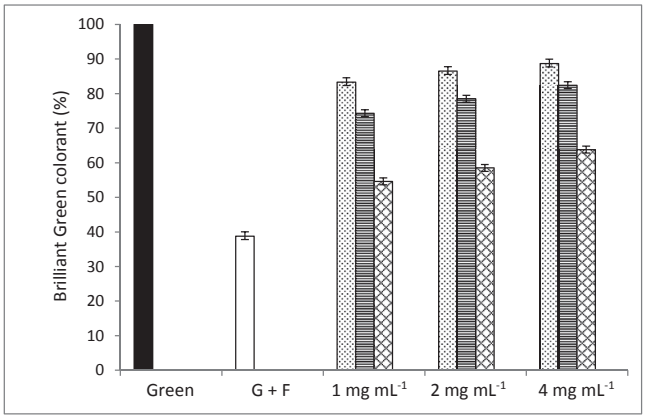


**Fig. 3.** Brilliant green alone (Green, ■) and initiated by Fenton reaction in the absence (G + F, □), and presence of different potential antioxidants at different concentrations (ascorbic acid, ▨; EPS W4, ▩; EPS W4*, ▤).

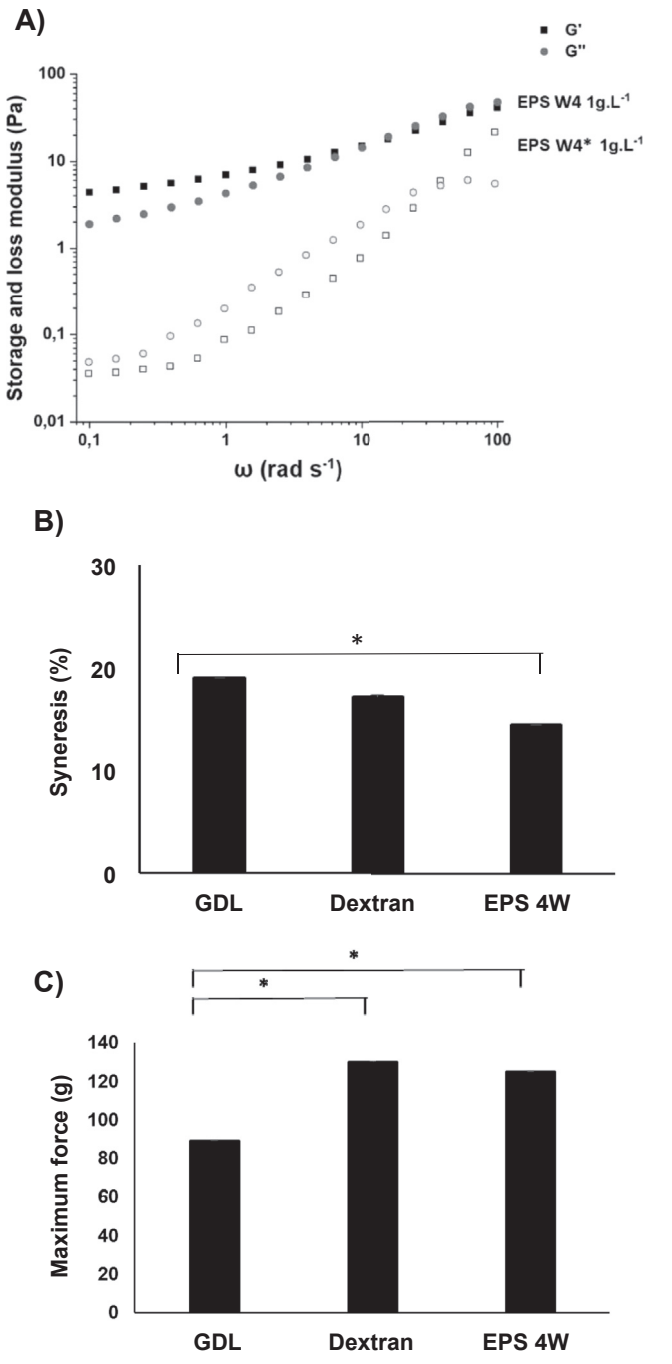


**Fig. 4.** Panel A, frequency dependence of storage modulus G′ and loss modulus G″ for 1 g $L^{-1}$ EPS W4 and EPS W4*; panel B, syneresis induced by centrifugation of 30 g of acidified milks in the presence of glucono-δ-lactone without polymer addition (GDL), with 1% commercial dextran of 500 kDa (Dextran) and 1% EPS W4 (EPS W4); Panel C, maximum force reached at gel compression of acidified milks in the presence of glucono-δ-lactone without polymer addition (GDL), with 1% commercial dextran of 500 kDa (Dextran) and 1% EPS W4 (EPS W4).

viscoelastic properties of EPS W4 (Fig. 4A) showed a higher storage modulus G′ and loss modulus G″ compared with EPS W4*, suggesting a more ordered polymeric structure. In addition, EPS W4 presented higher values of storage modulus G′ over the whole angular frequency range explored, revealing a more elastic than viscous structure. By removing proteins with TCA precipitation, EPS W4* presented opposite viscoelastic properties at low angular frequencies from 0.1 to 30 rad $s^{-1}$ since the structure presented higher values of loss modulus G" than storage modulus G'. The structure of EPS W4 was much more cohesive than that of EPS-W4*, particularly at low angular frequency. At a higher angular frequency, both EPS W4 and EPS W4* presented cohesive structures, implying that the EPS macromolecular chains mobility under a shear stress is highly affected by the presence of proteins interacting with the polysaccharide fraction (Ayala-Hernández, Hassan, Goff, & Corredig, 2009).

The ability of EPS to limit syneresis in milk acidified with GDL is presented in Fig. 4B. The results indicated that a texturising agent such as EPS W4 or industrial dextran had a significant and equal effect on decreasing syneresis of GDL acidified milk under centrifugation conditions (210×*g*). The control without EPS was significantly more susceptible to syneresis. The addition of EPS had a significant effect on the force required to penetrate an undisturbed acidified milk gel (Fig. 4C), however, this increase in gel strength was only significant comparing with the control yogurt, and displayed the same performance as the industrial dextran.

## 4. Discussion

In this study the EPS characterisation of a Klila indigenous *W. confusa* strain was first performed with attention given to the purification steps by partially removing or not a proteinaceous fraction from EPS by comparing EPS W4 and EPS W4*. Then, the roles of these EPS against oxidation reaction and in the texture of milk products were evaluated.

The purification procedure affects slightly molecular mass measurements for both EPS W4 and EPS W4*. Elevated molecular mass of approximately $10^7$ Da were detected for both EPS in our study, consistent with a previous work on *Weissella*-synthesised EPS (Adesulu-Dahunsi et al., 2018; Ahmed, Siddiqui, Arman, & Ahmed, 2012).

FTIR measurements were performed to complete the EPS characterisation. The results showed that the infrared spectra of EPS W4 and EPS W4* had approximately the same profile with a main absorption bands located around 3405.36 $cm^{-1}$, 2925.06 $cm^{-1}$, 1640 $cm^{-1}$, 1145 $cm^{-1}$, 1016 $cm^{-1}$. There was an increase in the peak intensity of the hydroxyl group and a significant decrease in the protein peak intensity for the EPS W4*. The precipitation of proteins (W4*) will cause a decrease in the quantity of proteins and consequently a modification of the structure of the macromolecular network, which results in changes in spectra.

The antioxidant activity of EPS depends on their origin, monomer composition, molecular mass, uronic acid, and protein content (Liu et al., 2017). Partial protein removal from the EPS structure led to a decrease in the antioxidant activity of EPS, which is consistent with previous work (Chen et al., 2010) demonstrating that EPS containing protein purified from *Alternaria* sp. have a higher DPPH scavenging activity. However, their hydroxyl radical scavenging activity was lower. These results suggest that part of the antioxidant activity may be due to cell envelope proteins or secretory proteins such as thioredoxin or glutathione reductase present in the EPS fraction. Indeed, the elimination of proteins has led to a significant modification of the macromolecular network with changes in the molecular mass distribution and structure of this macromolecular network that can influence its functions.

One of the most apparent and most frequently found defects in yoghurt is syneresis (Lucey, 2004), an unwanted phenomenon that results in the expulsion of whey from the protein matrix following the tightening of the gel, causing the whey to rise to the surface of the gel (Lucey, 2004). To avoid this problem, different types of thickeners and stabilisers are used (gelatin, alginate, starch, or pectin) (Duboc & Mollet, 2001; Lee & Lucey, 2010; Patel & Prajapat,

2013). However, these stabilisers do not have GRAS (generally recognised as safe) status and do not fulfil consumer requirements for products with fewer possible additives (Han et al., 2016). The use of EPS therefore offers the possibility of considering a new substitute for texturising agents.

The various physical (syneresis) and rheological properties (storage and loss moduli) of yoghurts are controlled by the structure and interaction of EPS with milk proteins. The presence of EPS made an additional contribution to the rheological and physical properties of milk acidified with GDL. W4 EPS increased apparent viscosity and had a significant positive impact on whey retention (low syneresis compared with control). These results are consistent with previous results (Gentès et al., 2011; Han et al., 2016; Hess et al., 1997) indicated that the use of EPS starter cultures reduced syneresis in yogurts. In addition, we found that the addition of the EPS W4 has the same texturising effect as that of an industrial dextran. Similar results have also been obtained by other studies (Guzel-Seydim, Sezgin, & Seydim, 2005; Han et al., 2016) that reported that the exopolysaccharides produced by LAB could improve the texture of yoghurt by interacting with the free water in the gel-like structure.

## 5. Conclusion

LAB Exopolysaccharides are considered natural antioxidants and have been reported as GRAS with beneficial effects on human health through the prevention and treatment of diseases. This work highlighted the possibility of using EPS in the industrial sector by revealing their properties in complex food matrices. These EPS can serve as substitutes for synthetic antioxidants which tend to form radical and/or reactive oxygen species that have been incriminated in the increase of certain diseases. The EPS isolated in our work have interesting texturising and anti-syneresis proprieties that would be beneficial in the dairy industry, where they can be used to reduce the production cost of certain foods by avoiding the use of additional non-GRAS additives to improve texture and better counter syneresis. Using EPS will also meet the demands of consumers for organic products.

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