

ORIGINAL ARTICLE

Influence of the exopolysaccharides of polyphenol-conditioned lactic acid bacteria on gut microecology and bacterial translocation

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The aim of this work was to assess in vivo prebiotic effects of exopolysaccharides produced by polyphenols extract-conditioned lactic acid bacteria. The polyphenolic content of *Thymus fontanesii* was extracted in water by sonication, with a yield of 41.5% and 156 mg equivalent of gallic acid/g. Gallic, caffeic, syringic, vallinic and carboxylic acids, catechin, and epicatechin were the important phenolic acids identified in the extract. Then, two dairy industrial strains *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were treated at different concentrations with the extract to improve exopolysaccharides production. *Streptococcus thermophilus* yielded more exopolysaccharides, thrice than control (826 mg/l vs 219 mg/l), in presence of 100 µg/ml (concentration of 0.01 mg/ml) of the polyphenolic extract. Besides, polyphenols had no significant effect on *Lactobacillus bulgaricus* for exopolysaccharides production. Last, the effects of *Streptococcus thermophilus* exopolysaccharides produced in presence of the polyphenols were evaluated on gut microecology composition on some bacteria and bacterial translocation in liver, spleen, kidneys, and lungs. The molecules shaped Wistar Rat gut microbiota in favour of beneficial lactic acid bacteria and in detriment of pathogenic bacteria, and prevented bacterial translocation. Therefore, the exopolysaccharides exhibited considerable prebiotic properties.

Keywords: Bacterial translocation; lactic acid bacteria; exopolysaccharides; polyphenols; prebiotic; *Thymus fontanesii*

Introduction

Gut microbiota keeps host organisms healthy and protects them from injurious ones. It may help them break down complex components from food, synthesize nutritive oligo-nutriments, antagonize pathogens colonization, and above all modulate immune defense. All those benefits relies on the richness, diversity, of this microbial diaspora, made up by hundreds species of bacteria (Scarpignato and Lanas, 2006). In fact, human inhabitant bacteria count 10 times more cells than human own cells, and the majority, almost one third, is located in gastrointestinal tract (Peterson et al., 2009). And 99% of them are anaerobes, from which are defined lactic acid bacteria (LAB) (Scarpignato and Lanas, 2006).

The protective functions of the microbiota include the barrier effect that prevents invasion by pathogens. The resident bacteria are the first line defense against colonization by transient microbes or opportunistic bacteria in the gut (Bourlioux et al., 2003). Dysbiosis, altered gut microecology, is an increasing issue with contemporary diets and antibiotic overuse (Belkaid and Hand, 2014). It is associated with gut related inflammatory, infectious diseases (Belkaid and Hand, 2014; Bourlioux et al., 2003). For example, *Clostridium difficile* overgrowth, produce toxins and causes pseudomembranous colitis, then it is incriminated in antibiotic-associated-diarrhea (Scarpignato and Lanas, 2006). The antibiotic use ruptures gut microbiota balance and permit overgrowth of this pathogen and leads to leaky gut (Berg, 1999).

Disruption of microbiota balance has premier effects bacterial overgrowth and leaky gut (Berg, 1999). Mucosal barrier dysfunction, rupture, permeability increase, is known as leaky gut and can cause translocation of a noticeable quantity of viable bacteria from gut into portal circulation (MacFie, 2005; Berg, 1999). Bacteria are then spread in sterile tissues such as liver, pancreas and lungs (Berg, 1999). The dissemination of these bacteria through the body may induce sepsis, shock, multiple organ failure, intestinal ischemia, intestinal obstruction, severe pancreatitis, acute cirrhosis and liver failure, or death of the host (MacFie, 2005). The relationship between microbiota and host is a delicate equilibrium that benefits host health. There are pharmaceutical or nutritional interventions that permit to modulate and optimize this symbiosis. One approach to

positively affect the composition of the intestinal microbiota and its metabolic activity is the ingestion of so called prebiotics (MacFie, 2005 ; Buddington, 2001). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and /or activity of one or a limited number of bacteria in the colon, and thus improve health (Gibson and Roberfroid, 1995). Many molecules have been reported with prebiotic properties.

Exopolysaccharides (EPS) of generally recognized as safe (GRAS) lactic acid bacteria (LAB) have received great interests as food-grade ingredients (Patel et al., 2012 ; Welman and Maddox, 2003). They possess important rheological (thickening, stabilizing, emulsifying and stabilizing) properties that serve many aspects in dairy industries (Caggianiello et al., 2016; Patel et al., 2012). In addition, they encompass beneficial properties for human health, such cholesterol lowering, antitumor, anti-ulcer, immuno-modulating activities as well prebiotic properties (Caggianiello et al., 2016; Patel et al., 2012). EPS have been found to enhance gastrointestinal colonization by beneficial bacteria and thus, play a significant role as prebiotics (Caggianiello et al., 2016 ; Welman and Maddox, 2003). However, marketing of these polymers is limited by their low level of production, which in most cases does not exceed a maximum of 3 g/l (Welman and Maddox, 2003). For instance, the highest known EPS-producer dairy starter *Streptococcus thermophilus* can only yield ~ 1 g/l (Wu et al., 2014). Many strategies have been explored to enhance EPS production. A major way to achieve high EPS levels during fermentation is to optimize culture conditions by enriching the medium with ingredients such as new carbon sources or interfering with microbial metabolisms (Welman and Maddox, 2003).

Polyphenols are plants secondary metabolites that are not absorbed in the small intestine but can be degraded by colonic microbiota to simpler and more easily absorbable bioactive compounds with potential physiological effects. They can act as potent antioxidants and have diverse effects on microbes. They appear to be effective antimicrobial agents against pathogens (Boubakeur et al., 2016 ; Borges et al., 2013) and to improve LAB growth and metabolisms (Boubakeur et al., 2016 ; Ye et al., 2012; Khalil, 2010). A polyphenol-rich diet may then improve the intestinal status and possibly attenuate oxidative stress and suppress inflammations (Jaksevic et al., 2013). These attributes express the potential prebiotic effects of those molecules. Furthermore, these compounds have technological aspects as they can enhance metabolism of LAB in dairy industry (Ye et al., 2012). The aim of this study is therefore to assess the influence of *Thymus fontanesii* polyphenols extract on the yield of two LAB-EPS and to evaluate their effects on animal model gut microbiota composition and bacterial translocation.

Materials and methods

Determination of the composition of the polyphenolic extract of *T. fontanesii*

T. fontanesii have been reported rich polyphenolic local pharmacopee plant that largely spread in Algeria. The leaves and flower are the most used part of the plant. In previous study (Boubakeur et al., 2016), four techniques were compared (overnight soaking at ambient temperature, brief heating-soaking, Soxhlet and sonication) and sonication yielded best with 41.5% recovery and polyphenols content 156 mg EGA/g of extract. The sonication extract was used in this study. The extract was filtered through filter-equipped syringe 0.45 µm, and 20 µl was injected directly without dilution in column ZORBAX Eclipse XDB-C18 (5 µl). The HPLC was coupled with UV-Detector YL9120 (254 nm). The phenolic acids standards were diluted in pure methanol.

LAB strains

Two local dairy industrial strains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* were investigated. Confirmatory identity of the two starter cultures have been conducted with API galleries (Strp and 50 CHI respectively). Aliquots are maintained at -20°C in 15 % glycerol (v/v) and overnight M17 agar subcultures were always used for tests.

EPS production

Inocula preparation

Bacteria were grown to exponential phase for 18 h in M17 broth and the optical density at 625 nm was adjusted by dilution in fresh media to a turbidity of 0.5 MacFarland standards to give an optimal inoculum size of ~10⁷ germs/ml.

Bacteria conditioning with polyphenolic extract

Bacterial cultures, at fixed density (~10⁷ germs/ml), are supplemented by the polyphenolic extract of *T. fontanesii* in increasing final concentrations : 0 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml and under the optimal fermentation conditions previously determined (data not published). In a previous study (Boubakeur et al., 2016) this extract has confirmed a remarkable activity on growth, aggregation and biofilm formation of *S. thermophiles* and *L. bulgaricus* at concentration of 100 µg/ml.

Isolation and quantification of EPS

EPS was isolated, purified according to procedure, with little alterations, described by Adebayo-tayo and Onilude (2008). The cultures were heated at 80°C for 15 min to deactivate hydrolyzing enzymes and to liberate cell-attached EPS and the cells pellet were removed by centrifugation at 5000 g/15 min. Trichloroacetic acid (TCA) was then used to inactivate residual EPS-

degrading enzymes. After 2 h, the preparation was ethanol-precipitated and centrifuged at 5000 g/15 min. Last, EPS was dissolved in water and quantified by phenol-sulfuric method of Dubois et al. (1956), with glucose as standard.

In vivo evaluation of prebiotic effects of EPS

Animal model and regimen

Albinos Wistar rats were gained from Pasteur institute in Algier and bred in animal facility at University of Tiaret. 16 Adult offsprings with average weight 250 g were enrolled in this study and divided into 2 groups including 8 rats each. Against negative control group (no treatment has been achieved), test group was force-fed 4 weeks with 82.6 mg of EPS produced under polyphenolic extract.

Rats sacrifice and organs microflora analysis

One female and one male rats, from each group, were anaesthetized with ether and sacrificed each week. Then, colon microbiota and bacterial translocation in liver and long were assessed on selective agar media (MRS, modified Columbia, Chapman, Hektoen and nutrient agar). Briefly, organs were collected, cleaned and grounded in Ringer's solution. Afterwards, they were filtered, diluted and plated for bacterial count (Lactobacilli, enterococci, staphylococci, total microflora).

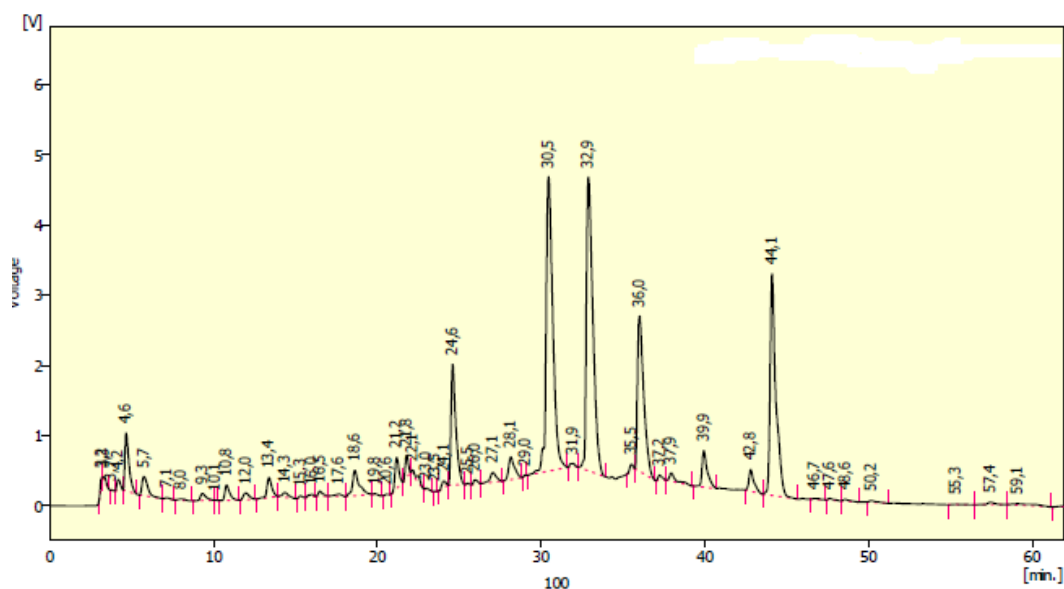
Statistical analysis

The results are reported as means \pm SEM and t-test was performed with significant p-value ≤ 0.05 .

Results and discussion

Phenolic acids composition of the extract of *T. fontanesii*

EPS production is improved in biofilm. In our previous study (Boubakeur et al., 2016), the extract of *T. fontanesii* improved growth and biofilm characteristics of *S. thermophilus* and *L. bulgaricus*. In fact, many works (Ye et al., 2012 ; China et al., 2012; Alberto et al., 2007) reported that polyphenols improve growth and technological attributes of LAB, including *S. thermophilus* and *L. bulgaricus*. The extract of *T. fontanesii* is very rich polyphenolic composition : Gallic acid, caffeic acid, Syringic acid, carboxylic acid, vanillic acid, catechin, and epicatechin (Figure 1). *T. fontanesii* extract have been reported rich polyphenolic contents, thymol, eugenol, carvacrol (Nabet et al., 2017; Dob et al., 2006).



Compound	Gallic acid	Caffeic acid	Syringic acid	Catechin	Epicatechin	Vallinic acid	Carboxylic acid
Std Reten. Time (min)	6.534 7.020	20.523	21.967	21.553	22,503 29,120	22,693 30,403	28,200
Resolution of standard	0,598	-	-	-	14,825	12,695	-
Resolution in sample	2,424	1,778	0,983	1,436	1,808	2,851	1,611

Figure 1. Phenolic compounds identified from 51 detections in the extract.

Influence of the polyphenolic extract on the EPS production

The rich polyphenolic extract improved *S. thermophilus* ability to produce EPS while having no effect on *L. bulgaricus* (Figure 2). Polyphenolic extract of *Thymus fontanesii* had optimal concentration (100 µg/ml) effect on *S. thermophilus* ($p \leq 0.05$). It yielded 826 mg/l compared to control 219 mg/l. This was very significant production of EPS, compared to general performance of EPS producer LABs (Wu et al., 2014; Welman and Maddox, 2003). Khalil (2010) reported that polyphenols adapted *S. thermophilus* develop dense surface attached EPS and increase milk viscosity (that may be associated to rheological properties of soluble EPS). The reduction of EPS production at 200 µg/ml may be attributed to growth inhibitory effect polyphenols may have on cells.

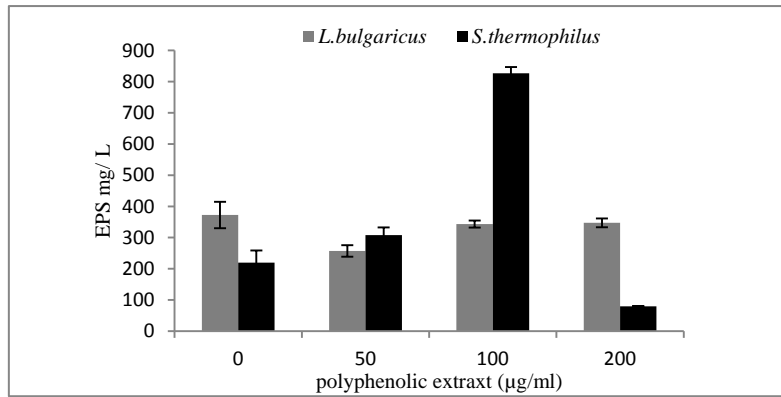


Figure 2. Influence of polyphenolic extract on EPS production.

In vivo prebiotic effects of EPS produced by *S. thermophilus*

The surface of the gut mucosa is particularly sensitive to the adhesion and colonization of pathogens from food, as well as commensal bacteria and probiotics. Bacterial adhesion to the intestinal epithelium affects the residence time and the ability of bacteria to exert their effects and functions in the intestine. The capacity to adhere to mucus and epithelial cells is considered an important selection criterion for probiotic strains. This ability varies depending on the strain, the cell surface properties such as the hydrophobicity and the production of slime (free EPS) (Van Halbeek, 1994; Bos et al., 1999; Dutta et al., 2012).

The results of the prebiotic effect of EPS, produced by *S. thermophilus* in presence of polyphenolic extract, on the diversification of the intestinal flora of the different groups of rats show a remarkable increase in the number of beneficial bacteria (lactobacilli) relative to the number of pathogens (enterococci and staphylococci) in the intestines (Figures 3 and 4). The effect is more important in colon than small intestine (Figures 3 and 4).

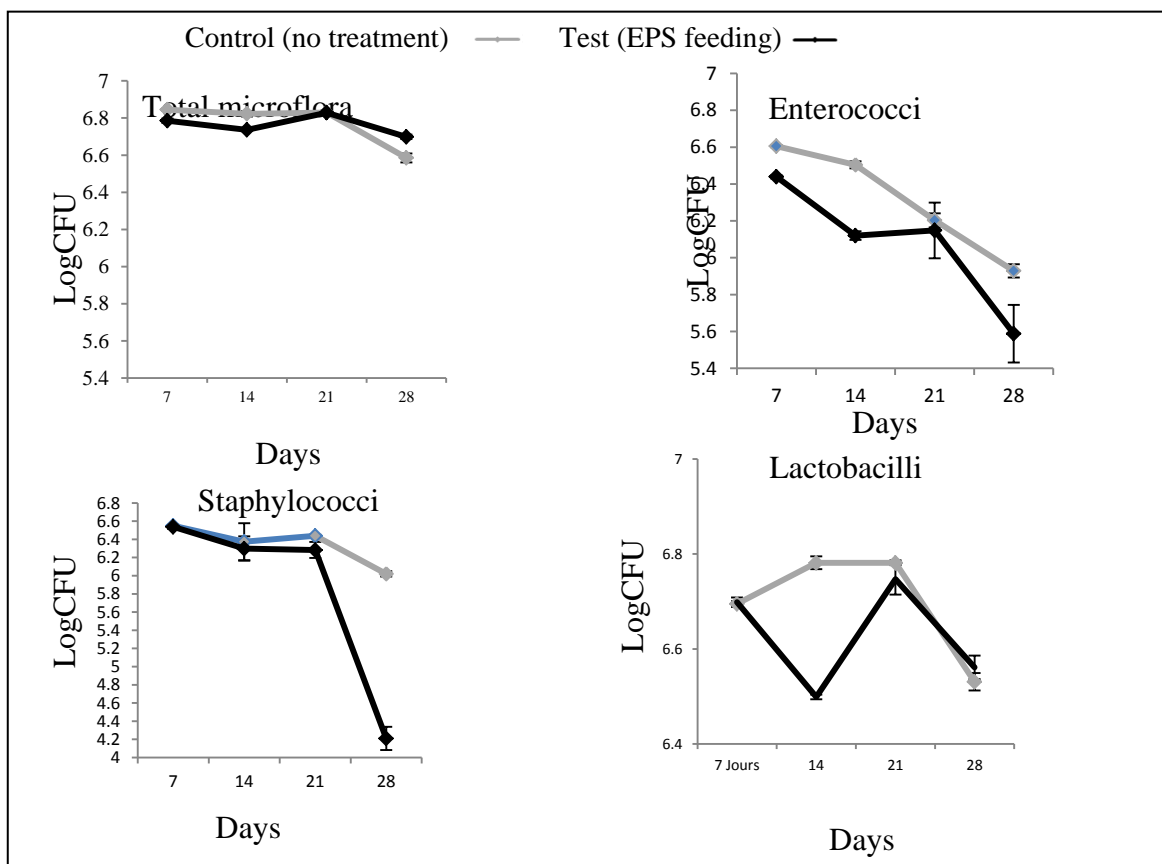
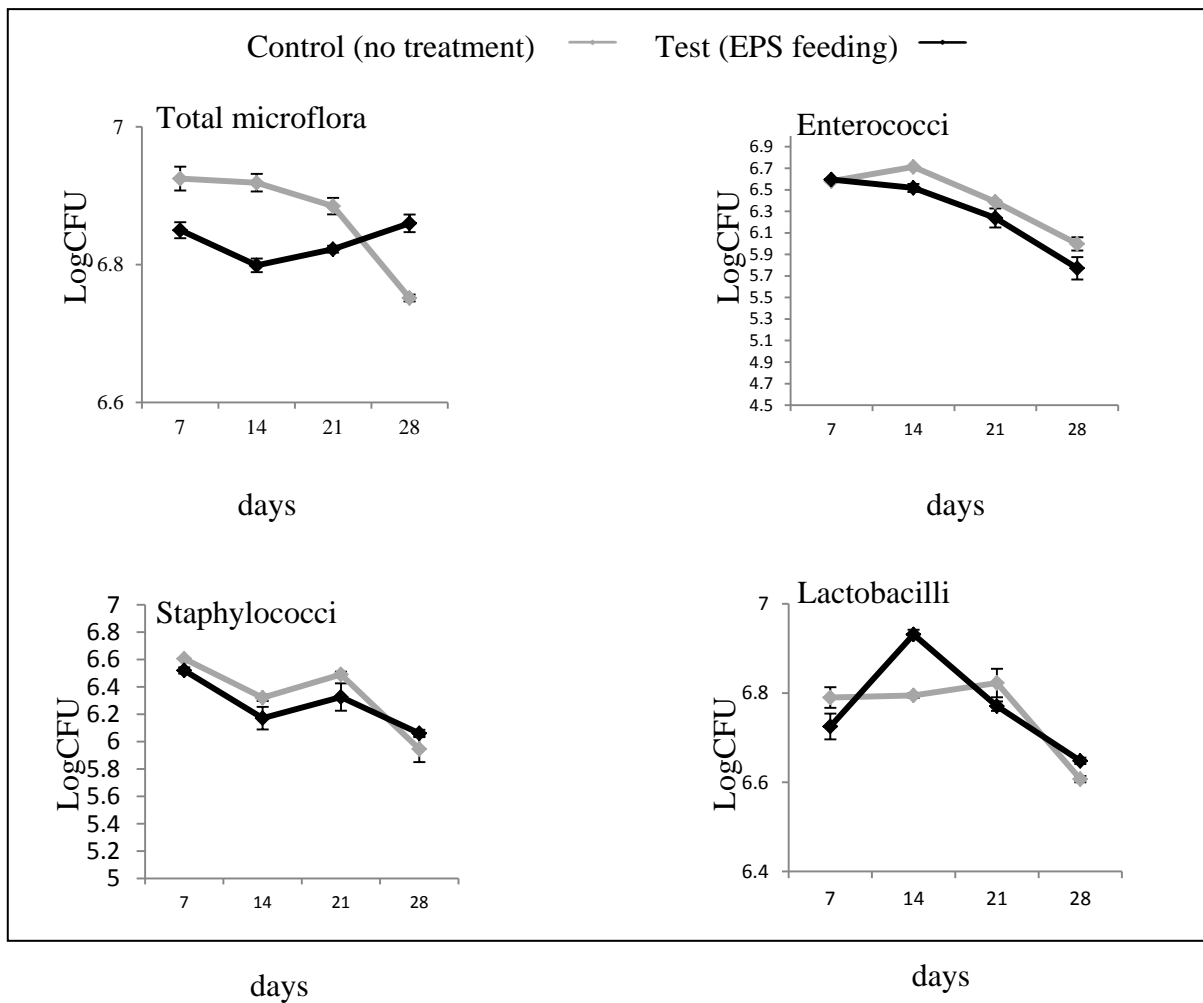


Figure 3. Effect of EPS on small intestine microbiota.

Some EPS of lactic acid bacteria were reported to have health benefits (Mugei et al., 2014), but few studies (Hongpattarakere et al., 2012; Ruas-Madiedo et al., 2006) have focused on their prebiotic effect *in vivo*. Hongpattarakere et al., (2012) in their study, using a model simulating the conditions of the colon, found a remarkable change in the composition of fecal flora under the effect of EPS from lactic acid bacteria. For their part, Ruas-Madiedo et al., (2006) reported EPS may prevent probiotics adhesion to human colon mucus. They also found that the effects may be EPS type and concentration dependent. According to genetic background or culture condition bacteria may produce EPS of different structures and properties. Besides, study conducted by Kim et al. (2009) showed an 87% decrease in biofilm of enterohemorrhagic *Escherichia coli* in the presence of 1.0 mg / mL of free EPS. The mechanism of action supporting this reduction in biofilm formation was a modification of the expression of genes related to the EPS production showing by transcriptome analysis. They also indicated that the EPS inhibited the initial attachment and autoaggregation of bacteria by attenuation and modification of the cell surface or by reducing cell - cell interactions.

**Figure 4.** Effect of EPS on colon microbiota.

Bacterial translocation to spleen, kidneys, liver and lungs occurred during the first week of treatment but was corrected from the second week of experimentation for the group of rats treated by EPS compared to the control group (Figures 5-8).

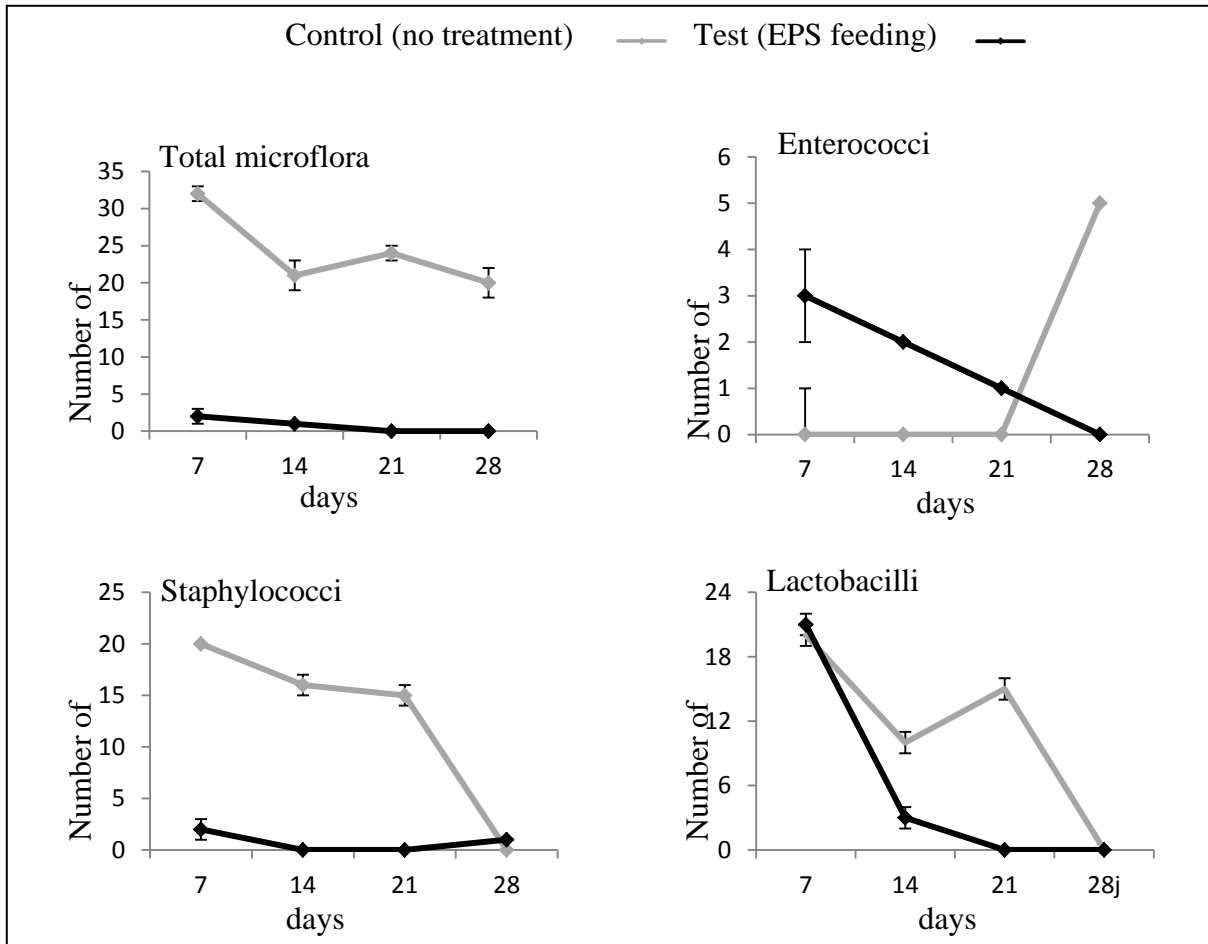


Figure 5. Effect of EPS on bacterial Translocation in lungs.

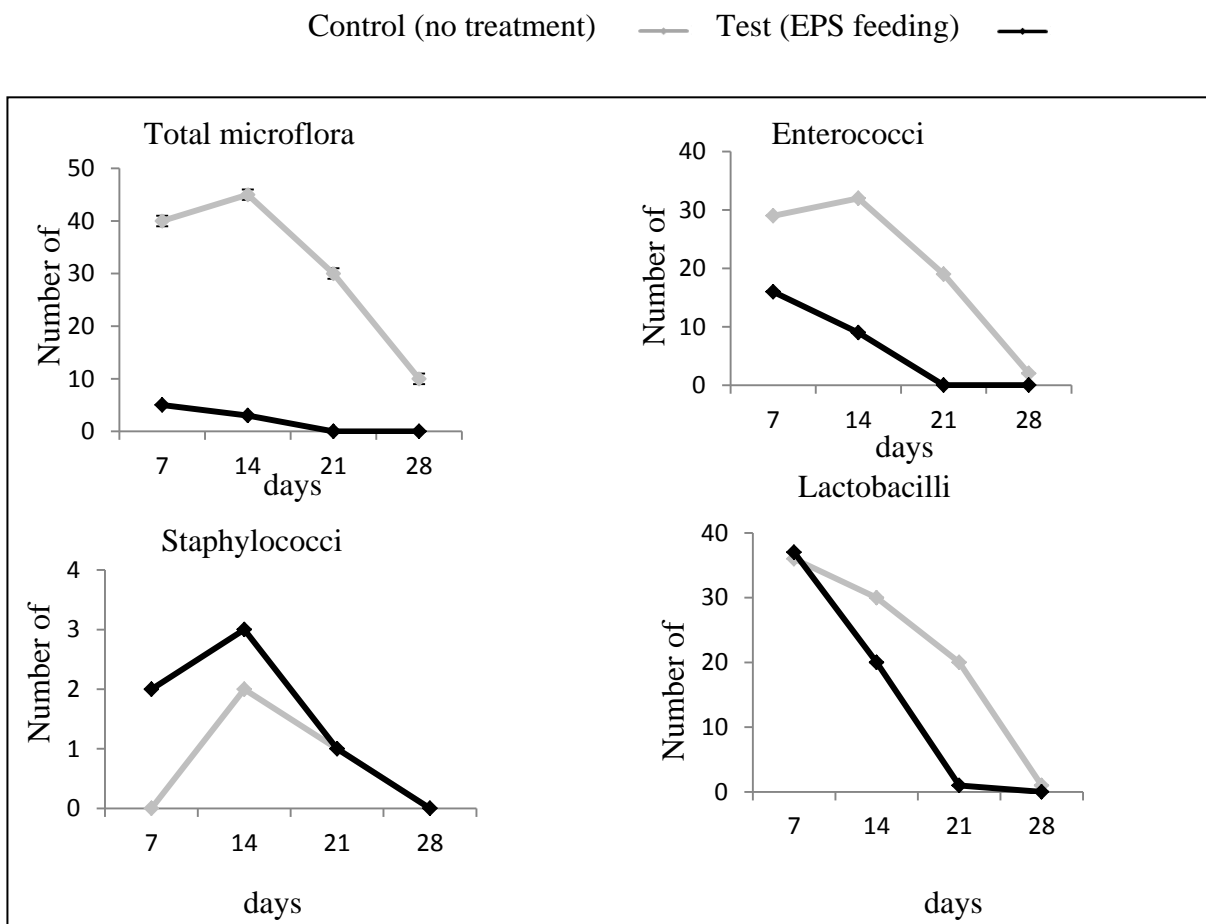


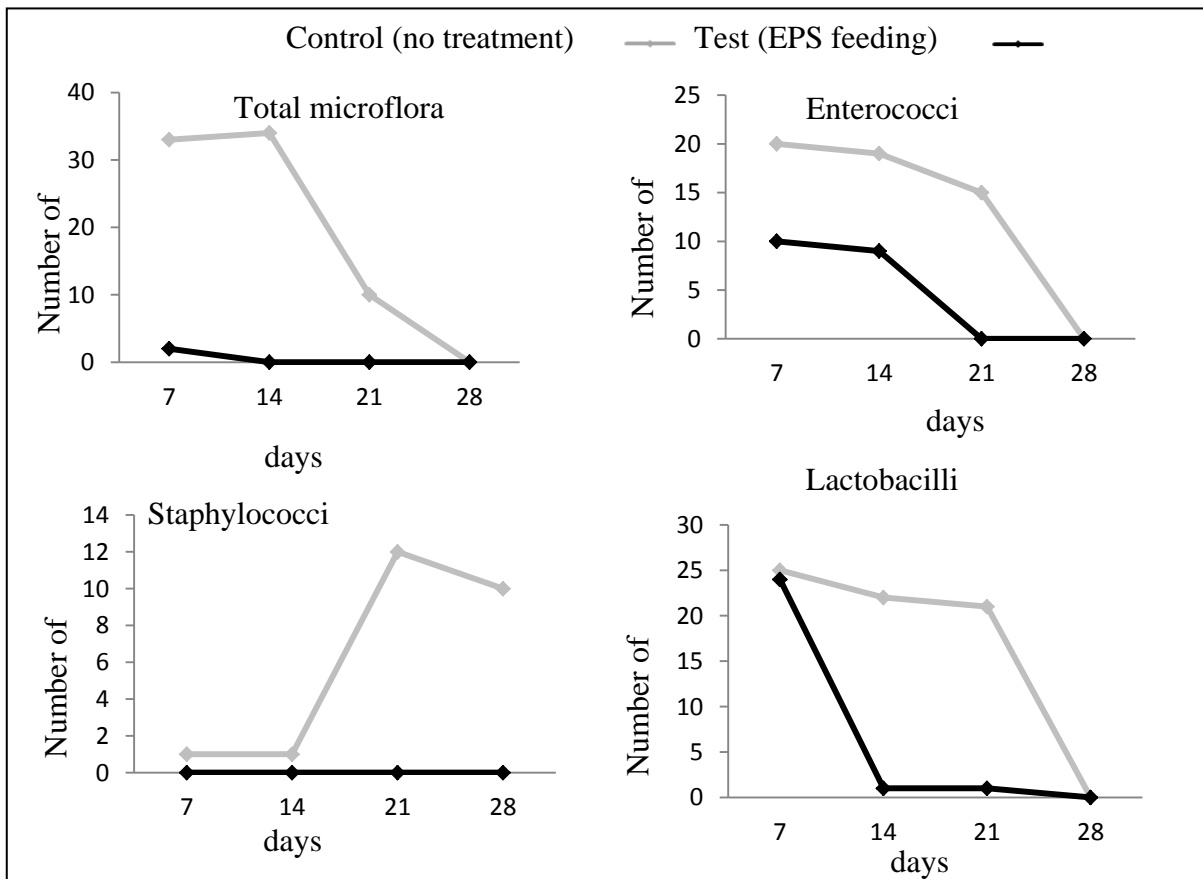
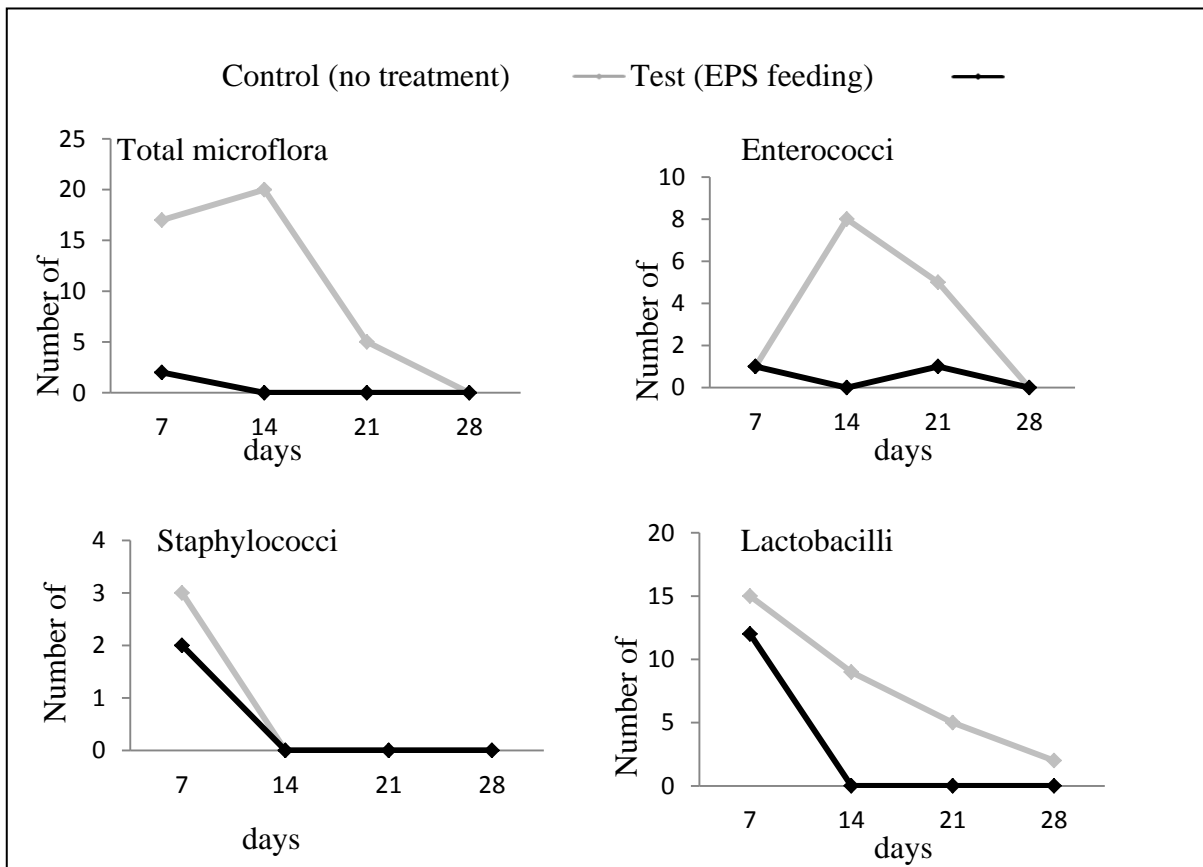
Figure 6. Effect of EPS on bacterial Translocation in liver.**Figure 7.** Effect of EPS on bacterial Translocation in kidneys.

Figure 8. Effect of EPS on bacterial Translocation in spleen.

EPS had beneficial effect in host microbial diversification and prevented bacterial translocation. There have been reported many mechanisms to explain those beneficial properties. Mao et al. (1996) found LABs and fibers or polysaccharides can reverse microbial disruption, decrease intestinal permeability, and as well as prevent the translocation of bacteria in chemical-induced enterocolitis rat models. Several studies have been carried on the in vitro prebiotic effect of EPS. Bello et al. (2001) showed that the population of *Bifidobacterium* was correlated with the concentration of EPS produced by *Lactobacillus sanfranciscensis*. In the same way, it has been proven that the EPS of lactic acid bacteria can affect the adhesion of pathogenic bacteria to the intestinal mucus and their colonization, changing the composition of the intestinal microflora of the host (Kim et al., 2009; Ruas-Madiedo et al., 2006; Looijesteijn et al., 2001). Gorska-Fraczek et al (2013) have shown that the EPS-producing probiotic bacteria can show distinct physicochemical properties on their walls and a greater adhesive capacity, they indicated that this effect depends very strongly on the structure of produced EPS. EPS is capable to bind to pathogens surface, thus altering their surface physicochemical properties (Ruas-Mudiedo et al., 2006). However, there are limited studies on the In vivo properties of LAB EPS and clear mechanistic studies are highly demanded.

Conclusion

In this study, the yield of EPS produced from *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* was improved in the presence of a polyphenolic extract of *T. fontanesii*, the yield of EPS from *S. thermophilus* reached up to 826 mg/l. In addition, the findings indicate that the EPS of *S. thermophilus*, produced under the effect of polyphenolic extract, may have a potential prebiotic effect. In this respect, these molecules, which have gained attention not only for their technological properties but also have thought to have prebiotic potentials, can be used as an alternative or additive agents to improve the probiotic bacteria activity (proliferation and colonization) and to prevent the pathogenic bacteria growth, colonization in and translocation.

Acknowledgments

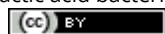
This work was realized in two laboratories: Laboratory of bioconversion, health safety and microbiological engineering/ University of Mascara, Algeria and laboratory of research for improvement and valorization of local livestock products/ University of Ibn Khaldoun, Tiaret, Algeria. The authors wish to thank the staff of these laboratories.

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